CROP PROTECTION PROGRAMME

Identifying Insect Vectors and Transmission Mechanisms for Banana *Xanthomonas* Wilt

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FINAL TECHNICAL REPORT

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1. EXECUTIVE SUMMARY

Banana *Xanthomonas* wilt (BXW), caused by the bacterium *Xanthomonas campestris* pv. *musacearum* (*Xcm*), is one of the major constraints to banana production in Uganda. Field observations suggest that the disease is spread primarily by insects through the male flowers. This study was initiated to establish insect vectors and transmission mechanisms of *Xcm* and to develop appropriate techniques to reliably isolate and identify *Xcm* from plants and insects.

We carried out an inventory of insects found on banana inflorescence, investigated possible sources of inoculum in banana plants, and identified insect species that carried the bacterium on their bodies and were thus possible vectors of the disease. The most abundant insects visiting banana flowers were stingless bee, *Plebeina denoiti* (Vachal) (Apidae), fruit flies (Drosophilidae) and grass flies (Chloropidae). These three insects were at least four times more abundant in male flowers of symptomatic plants in infected fields compared to male flowers in non-infected fields. Both female and male flowers from infected fields had almost twice as many insects as flowers from non-infected fields. Twice the number of insects visited the female flowers compared to male flowers.

We developed semi-selective media for isolation of *Xcm* from insect, soil and plants. We also developed rapid pathogenicity assays to confirm the identity of *Xcm*. On banana plants, *Xcm* was isolated from nectar, from cushion/scar exudates, and from ooze commonly found at the scars and between male flowers. Ooze had more bacterial cells compared to other sources. On insects, bacterial cells have been isolated from the stingless bee (*P. denoiti)*, honey bees (*Apis melifera),* fruit flies and grass flies that had been collected from male flowers of both asymptomatic and symptomatic plants. The number of bacterial colonies isolated from *P. denoiti* was two times higher than other insect groups. Field experiments demonstrated that *P. denoiti* and grass flies could transmit *Xcm* from infected to healthy plants. The results confirm that the practices of excluding insects from flowers by debudding currently carried out by farmers in Uganda are appropriate for the management of BXW.

2. BACKGROUND

In early 2001, a serious outbreak of banana Xanthomonas wilt (BXW), caused by the bacterium *Xanthomonas campestris* pv. *musacearum* (*Xcm*), was reported in Mukono district, Uganda. This devastating disease has quickly become the most serious threat to the banana production in the country. BXW can spread rapidly and is now present in eastern, central and northern Uganda. Recent reports of BXW in the Kabarole and Bushenyi districts place the disease on the edge of the country's primary banana growing regions in southwestern Uganda. If unchecked, the disease could cause massive losses in the Uganda's western districts, an area of intensive banana cultivation. The livelihoods of millions of Ugandan farmers are now at risk. BXW has also been confirmed in the Democratic Republic of Congo and in Rwanda, and it seems only a matter of time before it will enter Burundi, Kenya and neighbouring countries.

Bacterial wilt was initially reported in Ethiopia on *Ensete*. *Xcm* infection can result in severe losses in banana production due to early ripening and rotting of fruits (even in the absence of apparent external signs of the disease), and to wilting and death of banana plants. Ratoon crops arising from infected mats are severely diseased and often wilt before producing bunches or produce bunches with rotted fruits. All types of bananas appear susceptible,

although certain cultivars (e.g. ABB beer bananas) probably play a critical role in facilitating the spread of BXW.

Field observations suggest that the primary means of disease spread is by insect transmission through the male flowers. Secondary infection may occur through the roots or the use of contaminated tools. Wilting of floral bracts and the male bud, premature yellowing and rotting of fruits, and bacterial exudation from the peduncle are symptoms of infection through the inflorescence. Yellowing of leaves, yellow to pink bacterial exudates in the leaf sheaths, and plant wilting are symptoms of infection from soil-borne inoculum or from a previously infected pseudostem. BXW appears to be similar to Moko disease (*Ralstonia solanacearum*) of banana with respect to disease development, transmission and damage. Stingless bees, wasps and flies are believed to be important vectors of Moko disease, with infection commonly occurring through the moist cushions or scars of recently dehisced male flowers and floral bracts. However, because the disease is new, virtually no information is available about the vectors, infection courts, transmission epidemiology and biology of BXW. A good understanding of these factors is required for developing and targeting BXW management practices.

The Uganda National Banana Research Programme (UNBRP), in collaboration with other national and international partners, has undertaken an intensive BXW mitigation programme and countrywide awareness campaign. The primary objective of this campaign is to contain the disease and prevent its spread. The principal recommendation has been to protect banana plants by removing the male flowers to eliminate the most likely infection court. This recommendation has been drawn from the research and management of Moko disease in the Americas. However, the presence of similar groups of vectors and disease pathways for BXW remains to be confirmed.

Earlier DFID-funded research projects (R7567, R7529 and R7972) on banana IPM have developed an excellent network of collaborators from the grassroots level upward for dissemination of banana crop management practices.

3. PROJECT PURPOSE

The purpose of the proposed research is to generate strategic research information on biology, epidemiology and modes of transmission specific to BXW and pilot-level experiences of managing the disease with farmers with the aim of developing or adapting management methods to control the disease on a region-wide basis. More appropriate and reliable means of isolating and positively identifying the *Xcm* organism are essential to the success of this project and are contained within the proposal. The data to be obtained in the proposed activities are critical to support disease management efforts that are in progress on the ground.

4. RESEARCH ACTIVITIES AND OUTPUT

4.1 Potential of insects to transmit BXW

These experiments were conducted in farmers' fields in Mukono, Luwero and Mpigi districts where the disease exists under epidemic conditions. In each district we visited a sub-county in which the disease had been reported and surveyed five farms on which the disease was

present. All the farms belonged to the ABB Cultivar *Kayinja* (Pisang Awak) and were either abandoned or experienced very low management input. We sampled 10-15 randomly selected flowered plants on each farm. For each plant we recorded the development stages of the male and female parts of the inflorescence, time since flowering of first male flower, and presence or absence of disease symptoms.

4.1.1 Insect species that visit banana flowers

4.1.1.1 Inventory of insect species. We collected and identified insects from male and female flowers. A ladder was used to observe flowers without disturbing them. Then we put an insect net around the flower taking care not to disturb the flower and the insects on it. By grabbing the net close to its ring we captured the escaping insects in the net. The net was then carefully withdrawn so that insects could not escape out of it and to prevent flowers from falling into the net and contaminating the insects. The bottom of the net was dipped in a jar with chloroform vapour for 1 minute to knock out the insects. The insects were then emptied on a piece of paper and sorted according to recognizable morphospecies. Each group was placed in a bottle of alcohol, labelled and taken to the laboratory for further sorting and identification. Samples of collected insects were sent for identification to Prof. Dr. Dieter Wittmann, Institute for Agricultural Zoology and Bee Biology, Bonn, Germany.

Three species of stingless bees, *Plebeina denoiti* (Vachal) (Apidae), *Meliponula* sp. (Apidae) and an undetermined species (Apidae) were visitors of banana flowers (Table 1). Other insect species collected from banana flowers were fruit flies (Drosophilidae, undetermined spp), grass flies (Chloropidae, undetermined spp.), honeybees (*Apis melifera)*, beetles, and ants. Of the insects found, the stingless bee *P. denoiti*, fruit flies and grass flies were most abundant.

Table 1: Insect species and their frequency of occurrence on banana flowers

Insect species (Family/order)	Common name	Mean number/flower (±s.e)
Plebeina denoiti (Apidae)	Stingless bee	28.2±1.9 (321)[a]
Meliponula sp (Apidae)	Stingless bee	1.5±0.2 (6)
Undetermined species (Apidae)	Stingless bee	2.5±0.2 (105)
Apis melifera (Apidae)	Honey bee	3.3±0.3 (91)
Undetermined species (Chloropidae)	Grass flies	7.4±1.2 (183)
Undetermined species (Drosophilidae)	Fruit flies	10.7±1.2 (237)
Undetermined species (Hymnoptera)	Wasp	1.3±0.2 (6)
Undetermined species (Coleoptera)	Beetles	1.3±0.2 (16)
Undetermined species (Diptera)	Other flies	0.9±0.4(20)
Undetermined species (Hymnoptera)	Ants (all)	0.8±0.3 (45)

[a] The number of flowers from which insects were sampled are indicated in parenthesis.

4.1.1.2 Insect species visiting infected and healthy inflorescence. Sampling of insects that visit infected and healthy inflorescence was conducted as described above. In general, more insects were found on female than on male flowers from both infected and non-infected banana fields (Table 2). Among the frequent insect visitors, *P. denoiti*, grass flies and fruit flies were at least four times more frequently found in male flowers of symptomatic plants in infected field compared to male flowers in non-infected fields. Significantly more grass flies visited symptomatic male flowers compared to asymptomatic male flowers in diseased fields. About 93% more insects per flower were observed in asymptomatic plants in diseased fields

than in non-diseased fields. Most plants showing the disease symptoms had shed more than 15 bracts.

Table 2. Mean number (±s.e) of insects per female flower and asymptomatic and symptomatic male flower from infected and non-infected banana fields of exotic banana cultivar *Kayinja*

Insect (family)	Common name	Infected banana field			Non-infected banana field	
		Asymptomatic		Symptomatic		
		Female	Male	Male	Female	Male
Plebeina denoiti (Apidae)	Stingless bee	39.4±4.1	34.1±2.9	26.6±2.8	11.8±2.7	7.0±1.1
Undetermined species (Apidae)	Stingless bee	2.8±0.5	2.6±0.3	2.3±0.4	2.3±0.9	2.5±0.5
Meliponula sp. (Apidae)	Stingless bee	1.0±0.5	1.7±0.3	2.0±2.0	0.0±0.0	0.0±0.0
Apis melifera (Apidae)	Honey bee	3.6±0.6	3.3±0.5	3.3±1.2	2.0±0.6	2.8±1.2
Undetermined species (Chloropidae)	Grass flies	2.8±0.4	3.7±0.6	11.8±2.5	7.5±4.5	2.7±0.9
Undetermined species (Drosophilidae)	Fruit flies	14.1±4.1	9.3±1.6	10.2±1.8	21.3±6.2	2.7±1.2

***4.1.1.3 Insect activity on floral parts of banana plants infected with* Xcm.** To determine insect activity on floral parts of banana plants, samples of insects were taken from banana male flowers using an insect net at different times of the day (i.e. 8.00-10.00, 12.00-2.00 and 4.00-6.00). Sampling by direct observations of insect presence on the different parts of the male bud and behaviour in the field was also conducted.

For all insect species sampled from the male buds, insect activity was higher at 12.00-2.00 than at 8.00-10.00 and 4.00-6.00 (Table 3). During direct observation, 60-70% of stingless bees, *P. denoiti* were on the male flower or searching for nectar. Only 2-5% of stingless bees were observed on ooze, cushion and bract scar. Insect species such as honeybees and stingless bees flew from one part of the bud to the other (e.g. from bract scar to under the bract or male flower). Insects were also observed to fly from symptomatic to asymptomatic male buds.

Table 3: Mean (± s.e) number of insect visiting a *Xanthomonas campestris* pv. *musacearum* infected male bud of exotic banana cultivar Kayinja at different times of the day

Insect (family)	Common name	Mean number of insects		
		8.00-10.00	12.00-2.00	4.00-6.00
Plebeina denoiti (Apidae)	Stingless bee	17.0±2.4	37.0±2.7	9.9±1.0
Undertermined specices (*Apidae*)	Stingless bee	2.5±0.4	2.7±0.3	1.4±0.3
Undetermined species (Chloropidae)	Grass flies	4.5±0.8	8.2±1.7	7.8±3.5
Undetermined species (Drosophilide)	Fruit flies	7.2±1.5	13.1±1.7	5.7±1.1
Apis melifera (Apidae)	Honey bee	2.7±0.4	3.8±0.5	2.1±0.4

4.1.2. Location of Xcm *in flower parts*

Insects are likely to acquire *Xcm* from saps exuded from banana inflorescence. We conducted experiments to determine the sources of saps where *Xcm* might be present on banana flowers. We collected nectar from male flowers, ooze from naturally formed cushions/scars and in between flowers, and exudates from naturally formed cushions and fresh natural scars. One hundred µL nectar drawn from male flowers was serially diluted three times in a 1:9 ratio with sterile distilled water. Approximately 20 µL ooze was collected into a vial with 980 µL sterile distilled water then serially diluted seven times in a 1:9 ratio with sterile distilled water. A piece of sterile cotton wool, handled with sterile forceps, was used to wipe exudates from the fresh cushions/scars. The piece of cotton was then transferred to 1 mL distilled sterile water in a vial and then serially diluted three times in a 1:9 ratio with sterile distilled water. A drop (10 µL) of each dilution of each of nectar, exudate and ooze was spread uniformly on the surface of semi-selective medium (5-fluorouracil - Cephalexin Agar (FCA) containing (L^{-1}): 1 g yeast extract, 1 g glucose, 1 g peptone, 1 g NH_4Cl, 1 g $MgSO_4$. $7H_2O$, 3 g K_2HPO_4, 14 g agar, 40 mg cephalexin, 10 mg 5-fluorouracil and 120 mg cycloheximide). The plates were incubated for 5 days at 25-28^oC. The colonies that appeared light yellow, dome shaped, shiny, and circular were considered as *Xcm*. *Xcm* colonies were counted, and then transferred to a preservation medium for confirming their identity.

Table 4: Mean number of colonies of *Xanthomonas campestris* pv. *musacearum* (*Xcm*) isolated from different sources of sap of banana flower

Sap	*Xcm* colonies[1]
Nectar from male flowers	1.891×10^4 b[2]
Exudates from fresh cushion/scars	3.625×10^5 b
Ooze from cushions/scars and in between flowers	1.896×10^{11} a

[1]Number of colonies are expressed per mL for nectar and ooze, and per cushion/scar for exudates.
[2]Values followed by the same letter are not significantly different based on the LSD ($P = 0.05$).

Most *Xcm* was found in ooze, followed by exudates from cushion/scar, and the least in nectar (Table 4). The number of bacterial cells in ooze was significantly more than in nectar and cushion exudate. It was also noted that the numbers of bacterial cells available in these sources varied with stage of disease development (Table 5).

Table 5: Mean number of colonies of *Xanthomonas campestris* pv. *musacearum* (*Xcm*) isolated from different sources of sap collected from banana flowers at various stages of disease development

Stage of disease development	Average amount of nectar per flower (µL)	*Xcm* colonies[a]		
		Nectar of male flower	Exudates from cushion/scar	Bacterial ooze
No symptom	30.2	2.039×10^4	3.673×10^4	-[b]
Vascular necrosis	26.4	1.957×10^4	1.383×10^6	3.5926×10^{11}
1 bract wilted	16	1.338×10^3	.	5.8177×10^{10}
2-3 bracts wilted	2.7	2.557×10^4	.	1.3181×10^{11}
4 or more bracts wilted	0	-[b]	.	1.8681×10^{11}

[a]Number of colonies are expressed per mL for nectar and ooze, and per cushion/scar for exudates.
[b]No ooze or nectar available.

As symptoms became advanced, the amount of nectar in these plants declined, possibly due to plant–tissue death, but the number of bacterial cells recovered from available nectar increased. This was because plants were beginning to produce ooze, which later contaminated the nectar. It was noted that even the asymptomatic plants had *Xcm* in their nectar. Some agent, probably insects, may have contaminated the nectar with *Xcm*. Similarly, the number of bacterial cells recovered from cushions increased as the symptoms advanced. However, the number of bacterial cells recovered from ooze did not significantly change with advancement of disease symptoms.

Further investigations were carried out on ooze since it contained the maximum number of *Xcm* cells. When freshly formed, bacterial ooze flows but later dries with time. Ooze can be found flowing, dry or both flowing and dry at bract and flower scars or in between the male flowers. At different stages of disease development, we observed male inflorescence of banana to determine the site of ooze production and the status or consistency of the ooze.

Table 6: Percentage of plants with ooze of *Xanthomonas campestris* pv. *musacearum* in different parts of male inflorescence of banana and status of the ooze at different stages of disease development

Stage of disease development	Location of ooze			Status of ooze		
	Only cushions/ scars (A)	Only between flowers (B)	Both A and B	Only fresh (C)	Only dry (D)	Both C and D
No symptom	0.0	0.0	0.0	0.0	0.0	0.0
Internal vascular necrosis	100.0	0.0	0.0	42.9	28.6	28.6
1 bract wilted	0.0	0.0	100.0	0.0	0.0	100.0
2-3 bracts wilted	33.3	16.6	50.0	16.6	16.6	66.6
4 or more bracts wilted	36.8	0.0	63.1	0.0	31.6	68.4

None of the asymptomatic plants had any ooze (Table 6). All the plants showing vascular necrosis had ooze at the scars and the ooze was mainly fresh (43%). As symptoms advanced some of the ooze started flowing. For most plants that had four or more bracts wilted, this ooze could be found fresh and dry (68%) at both the scars and between the flowers (63%). There was generally a reduction in freshness of the ooze and an increase in its dryness as symptoms advanced.

This part of the investigation found that nectar, ooze and exudates could act as sources from which insects can mechanically acquire *Xcm* if they visit them. If they were to specifically acquire ooze mechanically, it follows that it becomes more difficult to acquire as symptoms advance because ooze dries up, but it could still be mobilised by rainfall.

4.1.3 Insect species that carry Xcm

***4.1.3.1 Identity of insect species carrying* Xcm.** To determine which insect species carry *Xcm*, and how much inoculum is picked up, insects were collected in nets as described above. On each farm, 5-7 plants were assessed and their disease status recorded. We sampled twice in two farms each in Mpigi, Luwero and Mukono. Five individuals of each insect species were collected and placed in a vial. In the laboratory, the insects were washed by suspending

them into 1 mL of 10%Yeast Peptone broth. The wash was then serially diluted 3 times (10^0, 10^{-1} and 10^{-2}) with sterile distilled water. A drop (10 µL) of each dilution was spread plated on to the FCA medium and incubated at 25°C for 5 days. At the end of this period, plates were examined for growth of *Xcm*-like colonies and the number of bacterial colonies per five individuals of each insect species determined. Purified colonies were transferred to the preservation medium for confirmation by pathogenicity tests.

Colonies of *Xcm* grew from washings from stingless bees (*Plebeina denoiti* and an undetermined species), honeybee *Apis melifera* (Apidae), grass flies (Chloropidae, undetermined species) and fruit flies (Drosophildae, undetermined species) that had been collected from both asymptomatic and symptomatic plants (Table 7). More contamination was observed in insects collected from symptomatic plants compared to asymptomatic plants. *Plebeina denoiti* carried more inoculum than all other insects. The fact that *Xcm* was isolated from insects collected from asymptomatic plants indicates that the insects can readily carry the bacteria from flowers on diseased plants to flowers on non-diseased plants.

Table 7: Mean number (±s.e) of *Xanthomonas campestris* pv. *musacearum* (*Xcm*) colonies isolated from insect vectors collected from asymptomatic and symptomatic flowers of exotic banana cultivar Kayinja

Insect (family)	Common name	Mean number of *Xcm* colonies per insect [a]	
		Asymptomatic plants	Symptomatic plants
Plebeina denoiti (Apidae)	Stingless bee	1645±1197 (3)	6073±3274 (25)
Undetermined species (Apidae)	Stingless bee	2637±977 (5)	1368±3274 (9)
Undetermined species (Chloropidae)	Grass flies	-[b]	2543±1963 (7)
Undetermined species (Drosophilidae)	Fruit flies	1647±1197 (3)	2398±1294 (11)
Apis melifera (Apidae)	Honey bee	-	5056±3275 (6)

[a] Number of insects that were positive for *Xcm* in parenthesis.
[b] No insect was positive for *Xcm*.

***4.1.3.2 Proportion of insects carrying* Xcm.** Insect species were collected from male buds of symptomatic plants. Insect species that had proved positive for carrying *Xcm* were picked with an aspirator from the net and placed individually in vials. The vials were taken to the laboratory to isolate the bacteria following the methods described in section 4.1.3.1. The proportion of insects from which *Xcm*-like colonies grew was calculated. Purified colonies were transferred to the preservation medium for confirmation by pathogenicity tests. A higher proportion of the stingless bee *P. denoiti* and the Drosophilids carried *Xcm* compared to other insect species (Table 8).

Table 8: Mean percentage of insects from which *Xanthomonas campestris* pv. *musacearum* (*Xcm*) grew on 5-fluorouracil -Cephalexin Agar medium

Insect species (Family)	Total number insects observed	*Xcm*-positive insects (%)
Plebeina denoiti (Apidae)	71	21.1
Undetermined species (Apidae)	12	0.0
Undetermined species (Chloropidae)	39	5.1
Undetermined species (Drosophilide)	58	19.0
Apis melifera (Apidae)	14	0.0

***4.1.3.3 Insect parts carrying* Xcm.** Stingless bees, *Plebeina denoiti*, were captured from diseased plants using nets as described above. To avoid cross contamination, each insect was picked with a sterile forceps after immobilisation and placed on a new sheet of paper. The head, abdomen and thorax were carefully separated and examined for surface contamination with bacteria. Appendages of each part were not removed. Forceps were heat-sterilised before handling a new insect part to avoid cross contamination. Each part was placed in a vial and taken to the laboratory where they were washed in 200 µL of 10% Yeast Peptone broth. Without further dilution, triplicate platings of 10 µL was done on FCA medium, and incubated at 25-28ºC for 5 days. Colonies resembling morphologically to *Xcm* were counted and preserved to confirm their identity by pathogenicity tests. All three insect body parts harboured *Xcm*. Similar number of *Xcm* colonies were obtained from thorax and ab ($\chi 2$ =2.94, d.f.= 1, P = 0.086), which were significantly more than colonies obtained from the abdomen ($\chi 2$-test, $P < 0.05$) (Table 9).

Table 9: Mean number (±s.e) of colonies of *Xanthomonas campestris* pv. *musacearum* (*Xcm*) isolated from the different parts of the stingless bee (*Plebeina denoiti)* captured from symptomatic male buds of banana

Insect body part	*Xcm* colonies per insect part
Head (n = 5)[a]	524±185
Thorax (n = 10)	581±193
Abdomen (n = 13)	180±175

[a] n = number of insects parts that were positive for *Xcm* and from which isolations were carried out.

4.2 Laboratory identification of *Xcm* isolates

4.2.1 Improved selective media for Xcm.

Prior to the start of this project, work carried out by NARO with support from the Gatsby Charitable Foundation (GCF) had concentrated on finding growth media on which colonies of *Xcm* developed quickly, to the point where they could be identified with reasonable confidence from distinctive features (yellow pigmentation, dome-shaped, translucent, glistening morphology) before becoming overgrown by other bacteria commonly isolated from diseased plant materials. Colonies were found to develop slightly faster on media based on bacteriological peptone or tryptone plus yeast extract than on nutrient agar or yeast extract alone. Inclusion of 1% or 2% glucose produced larger colonies that could be distinguished

more rapidly, although this did not shorten the time to their first appearance and also encouraged overgrowth by other, slime-forming, bacteria. Yeast extract (3-5%), peptone (5%), and agar (1.0-1.5%), usually with addition of glucose (1%; referred to as Yeast Peptone Glucose Agar or YPGA), became the standard growth medium. Inclusion of crystal violet (5-10 μg mL^{-1}) increased selectivity by inhibiting Gram-positive bacteria and some fungi, but appeared to reduce the rate of growth of *Xcm*, and early recognition of colony characteristics was more difficult.

Provided sensible aseptic precautions were taken, these media were adequate for isolation and recognition of *Xcm* from the interior of diseased banana plants that had not become moribund or extensively colonised by secondary invaders or saprophytes. However, isolation failures through overgrowth of 'contaminating' bacteria (and sometimes fungi) were quite common, especially from samples stored too long before isolation. YPGA was also insufficiently selective for investigation of more challenging microbial environments, such as dead plant materials, and soil. The suitability of this relatively non-selective medium for studies on the ecology of *Xcm* on plant surfaces and insects had not been extensively tested. It was shown to be possible to isolate *Xcm* from parts of banana flowers, including nectar and bacterial ooze collected from floral surfaces, and from insects. However, overgrowth by other organisms was a frequent problem, not to mention possible confusion with other bacteria having similar colony characteristics to *Xcm*.

In preliminary work, a few commonly used selective agents were tested against a single isolate of *Xcm*, which was found to be relatively insensitive to cephalosporin (>100 μg mL^{-1}) and cetrimide (100 μg mL^{-1}), moderately sensitive to amoxycillin (50 μg mL^{-1}) and neomycin (50 μg mL^{-1}) and highly sensitive to polymyxin B (<10 μg mL^{-1}). Of these antibiotics, polymyxin B has probably been the most widely used for the selective isolation of plant pathogens (particularly *Pseudomonas* and its allies) from other Gram-negative bacteria associated with plant materials (notably *Erwinia* and *Pantoea*) – so it was disappointing to find that it could not be used for *Xcm*.

Further tests on a range of antimicrobials were carried out by Dr. Leena Tripathi (IITA/NARO banana biotechnology programme), using a standard reference isolate of *Xcm*, from which the cephalosporin derivatives cephalexin or cephalozin (at 25 μg mL^{-1}) and cycloheximide (150 μg mL^{-1}) were chosen as selective agents non-inhibitory to *Xcm* but likely to be inhibitory to a broad spectrum of other bacteria and fungi. However, these antibiotics were still insufficiently selective to control growth of certain 'problem' bacteria, especially from microbially diverse environments such as decaying banana materials and soil. Accordingly, two approaches were tried (alone and in combination) to develop more selective media: (1) investigation of further antimicrobials for selective inhibition of growth of bacterial contaminants; and (2) investigation of culture conditions (growth substrates, osmotic potential) that might favour growth of *Xcm* over that of contaminating bacteria. The first approach was guided mainly by M. Mwangi and the second by S. Eden-Green.

***4.2.1.1. Use of selective agents to improve selective media for* Xcm**. The objective was to develop a medium that could selectively recover *Xcm* colonies from soil, plant debris and insect vectors. Although the routinely used non-selective medium YPGA is suitable for rapid growth of *Xcm*, the medium has disadvantages in that other contaminating bacteria grow rapidly and overwhelm *Xcm*, hence making it difficult to determine presence of *Xcm* and enumerate its populations.

Nineteen additional compounds were evaluated for selectivity against contaminants. Eight compounds including Amoxicillin, Erythromycin, Phophomycin, Rifampicin, Polymixin sulfate, Methyl violet 2B, Novobiocin, Neomycin sulphate and Bacitracin suppressed or inhibited *Xcm* and were excluded. Boric acid 0.03%, methyl green 2ml L^{-1}, potassium tellurite 15 $mg^{-1}L$ and TZC 2 mL $/L^{-1}$ (1% solution) did not add value to the medium and were excluded. Aztreonam, Nitrofurantoin, Tobramycin, Trimethoprim and Tyrothricin each suppressed only one main bacterium isolated from flowers and nectar. 5-fluorouracil suppressed many Gram-negative, and most importantly, two of the major fast-growing isolates in plant tissues. Cephalexin suppressed a wider range of organisms than cefazolin, and was therefore selected.

An improved semi-selective medium was thus developed, designated Cellobiose-Cephalexin Agar (CCA). containing (L^{-1}): 1g yeast extract, 1 g glucose, 1 g peptone, 1 g NH_4Cl, 1 g $MgSO_4$. $7H_2O$, 3 g K_2HPO_4, 1 g beef extract, 10 g cellobiose, 14 g agar, 40 mg cephalexin, 10 mg 5-fluorouracil and 120 mg cycloheximide. Including cellobiose as a carbon source further enhanced selectivity.

The semi-selective medium was tested for selectivity with soil, plant and insect samples. To determine selectivity when isolating from vectors, insects were trapped from banana flowers using a net and immobilised by briefly exposing to chloroform. Up to five insects of each family were separately placed in a test tube and washed using 1 mL sterile water in the lab. The resulting suspension was immediately spread (20 µL per plate) on three replicate plates each of YPGA and CCA. To determine selectivity with soil samples, a 0.1 mL suspension of *Xcm* cells was added in 2 g of moistened non-sterile soil obtained from banana farms. The infested soil was suspended in 20 mL water, the soil suspension shaken, 1 mL of the suspension serially diluted, and 20 µL of the dilutions spread on YPGA and CCA. To test selectivity with plant tissues, rotting banana fruit and stem tissues were macerated in water (10 g tissue in 100 mL water). One mL of the resulting suspension was serial diluted, and 20 µL spread on CCA and YPGA. The inoculated plates were incubated for 48 and 120 h for YPGA and CCA, respectively, and colonies counted. Efficiency of the media to selectively isolate *Xcm* was calculated as follows: Selectivity (%) = (Number of *Xcm* colonies on medium × 100)/ Total number of colonies on medium.

When isolating *Xcm* from insects, CCA had a selectivity of 57–73% depending on species, which was significantly higher than 4–17% selectivity achieved on YPGA (Table 10). *Plebeina denoiti* from infected plants had large numbers of *Xcm* cells while Drosophilide and *A. melifera* carried fewer cells. With soil samples, CCA had a selectivity of 82%, which was significantly higher than 42% achieved with YPGA. Growth of *Xcm* on YPGA was difficult to detect after more than 48 h incubation due to presence of fast-growing contaminants with slimy growth that overran *Xcm* rapidly. When isolating *Xcm* from infected but non-rotted stem tissues, both CCA and YPGA exceeded 95% selectivity. Selectivity of CCA was on an average 60% and 84% when infected rotted banana fruits and banana stems, respectively, were sampled. No bacteria produced slimy growth on CCA compared to extensive slime production exhibited by many contaminants on YPGA.

Table 10: Selectivity (%) of YPGA and Cellobiose-Cephalexin Agar (CCA) and Yeast Peptone Glucose Agar (YPGA) medium for isolation of *Xanthomonas campestris* pv. *musacearum* from insects suspected to transmit the pathogen

Location	Insect	Medium	
		YPGA	CCA
Mukono	Stingless bee – small (*Plebeina denoiti)*	15.1[a] ± 11.1[b]	73.6 ± 18.8
	Stingless bee – big (Undetermined species of Apidae)	8.4 ± 2.7	62.2 ± 12.0
	Honey bee (*Apis melifera*)	4.0 ± 1.2	48.9 ± 5.4
Mpigi	Stingless bee – small (*Plebeina denoiti)*	16.7 ± 11.0	64.3 + 6.2
	Honey bee (*Apis melifera*)	12.1 ± 2.8	57.1 ± 2.4
	Fruit fly (Drosophilide)	17.2 ± 11.3	74.8 ± 9.8

[a] Values are average of three replicate samples. Stingless bees per sample n = 5; honey bee n = 2; Drosophilide n = 3.
[b]Standard deviation.

In addition to *Xcm*, other bacterial species were frequently associated with insect vectors. The type of bacterial species and their populations differed between the insect species and sampling regions, possibly due to ecological differences. These associated bacteria could have implications in control of *Xcm* because they occupy the same niche on the insect surfaces and probably also on the plant floral parts. Isolates that grow rapidly and, therefore can compete with *Xcm* for nutrients and space, could be exploited in biocontrol strategies, targeting insects as agents to disseminate the biocontrol agent.

The improved semi-selective medium is being used in studies on ecology and survival of *Xcm* and will benefit NARS scientists in other countries facing the threat of BXW in their territories.

4.2.1.2. Use of growth substrates to improve selective media for Xcm.

Work by Simon Eden-Green (SEG) was carried out during two visits to Kawanda during April-May and June-July 2005 and details are given in Appendix 1. Two experiments were carried out during the first visit, using yeast peptone agar and a basal mineral salts (ARJ) agar medium loaded with 'depots' of various carbon/energy sources selected partly from results of Biolog tests (a commercially available metabolic profiling system) previously carried out at CABI Bioscience, UK. Plates were prepared with and without a low concentration of yeast extract (to provide a source of growth factors needed by many strains of *Xanthomonas*) and were inoculated with two isolates of *Xcm* and isolates of two 'problem' contaminating bacteria. Although several plates suffered from contamination, it was possible to draw the following conclusions:

- *Xcm* grew very poorly in the absence of yeast extract and probably has an obligate requirement for growth factor(s) such as biotin or nicotinic acid.
- It is probable that none of the carbon sources tested was present at sufficiently high concentration to stimulate growth.

From these limited results, further tests were recommended with aconitate, cysteine, methionine, acetate and salts of other available organic and amino acids. It also seemed clear

that a key to reducing overgrowth by contaminating bacteria was to keep the carbohydrate concentration in the growth medium as low as possible, without reducing the rate of growth of *Xcm*. In previous experiments (carried out in 2004), colonies of *Xcm* appeared quickly on 1% tryptone agar, even without added yeast extract or carbohydrate, so this might be a better starting point for developing a selective medium than YPA. Further experiments were suggested to establish optimum concentrations (as simply measured by speed of appearance and rate of growth in colony diameter) of tryptone with and without added yeast extract, then to test for optimum concentrations of additional growth substrates selected from carbon source utilisation tests on a minimal salts medium such as ARJ.

During the second visit (also summarised in Appendix 1), growth of a single recent isolate of *Xcm* was compared on yeast tryptone agar (YTA) and on ARJ medium supplemented with one-tenth strength YTA. In this case, carbon sources were incorporated into the agar medium as a gradient of concentration up to 1% (w/v). Results showed that best growth, in terms of time to reach recognisable colony morphology, occurred on media containing carbohydrates, especially the disaccharides cellobiose and trehalose. However, carbohydrates encouraged slime production, and hence overgrowth, by contaminants. These tests confirmed that *Xcm* (at least the one isolate tested) utilised aconitate and xylose. These compounds are less widely utilised by other organisms so might provide a basis for further work on selective media. However, they would have no advantages on the basal media used here as they did not improve the rate of initial colony appearance over the water controls.

Another approach tested was the ability of *Xcm* to grow in media with low water activity (high osmotic pressure). Plant nectar can contain quite high concentrations of sugars and this can be inhibitory to the growth of many bacteria – a fact that has been used as the basis for a selective medium for at least one plant pathogenic bacterium (*Erwinia amylovora*) that has an epiphytic phase in nectar. Growth medium (YTA) were prepared containing different concentrations of sucrose or sorbitol as osmotica and inoculated with two recent isolates of *Xcm*. Both media gave similar results. Growth of *Xcm* was completely inhibited at 15% sorbitol or 30% sucrose and partially inhibited at 10% sorbitol and 20% sucrose (these sorbitol and sucrose concentrations have similar molality and hence osmotic pressure/water activity). At the lower concentrations, growth of *Xcm* was sparse indicating that plating efficiency would be poor and that it was unlikely that these sugar concentrations, close to the limits for growth of *Xcm*, could be used as the basis for a selective medium. At lower concentrations of both carbohydrates, it is likely that many contaminating bacteria would produce abundant slimy growth that would also quickly overgrow *Xcm*.

4.2.2 Tests to purify and confirm **Xcm** *identity.*

Like most *Xanthomonas* species, *Xcm* produces a polysaccharide slime and non-diffusable yellow pigment on common bacteriological culture media, resulting in production of distinctive yellow, translucent, glistening (shiny), dome-shaped colonies that are relatively easy to distinguish from contaminating organisms of other genera. For most purposes a presumptive identification to *Xanthomonas* can thus be based on colony characteristics and for most purposes, especially when isolating from diseased banana tissues, it will be safe to assume that *Xanthomonas*-like colonies will be *Xcm* provided these conform to typical characteristics observed in subcultures that have been purified, subcultured and incubated under standard conditions.

Difficulties may arise with cultures obtained from environments where other xanthomonads may be present such as plant, and possibly insect, surfaces. The problem here lies at the sub-genus level (species and pathovar) where conventional bacteriological tests are unreliable or completely lacking in specificity. Molecular tests, based on detection and differentiation of ribosomal DNA, hypersensitive response and pathogenicity (hrp) genes or other parts of the genome, can be used to identify specific pathovars of *Xcm* and indeed these have already been used by staff at Kawanda. However, these would be both expensive and time consuming for routine use and even then samples of the results would need to be validated by pathogenicity testing, especially should any variability arise. Serological tests, using poly- or monoclonal antibodies specific to *Xcm*, provide alternative methods for bacterial identification. Preparation of polyclonal antisera (in rabbits) was commenced by staff at Kawanda late in 2004 and by IITA in 2005. Although this lies outside the scope of the present project, both groups have had some success and further tests are in progress to determine the activity and specificity of the antibodies and to devise simple tests suitable for routine diagnosis. However, pathogenicity to banana will always be the definitive way to confirm the identity of *Xcm* and, especially given the ready availability of banana plants at Kawanda, this was felt to be the best approach to definitive identification for the purposes of this project.

The output was thus delivered by ensuring that there were robust and standardised techniques for isolation and for purification of *Xcm* so that presumptive identification could be carried out by examining pure cultures on standard culture media maintained and observed under standard conditions. This was followed by preservation of pure cultures (in practice, given the number of isolations involved, a sample of pure cultures) for subsequent definitive identification by pathogenicity testing in banana. At the start of this project, generally good laboratory practices were already in operation in the bacteriology laboratory at Kawanda and, in an effort to strengthen these, a consultative document was produced to agree standardised procedures that would help to ensure successful isolation, purification and preservation of isolates of *Xcm* (see Appendix 2).

4.2.2.1 Preservation of the bacterial isolates. All suspected isolates of *Xcm* were preserved in glycerol stocks in duplicates. The bacterial colonies of each isolate were purified by sub-culturing on YPGA medium. A loopful of the pure colonies was suspended in YPG broth and incubated for 48 hours at 25-28^{o}C. The glycerol stocks were prepared by mixing glycerol and YPG broth in a 1:1 ratio. This mixture was autoclaved for 15 minutes at 121^{o}C. One mL of the growth in the YPG broth was drawn into a vial and centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded and the cells suspended in 1 mL of cool glycerol stocks in a screw-capped vial. These were stored at –20^{o}C awaiting confirmation of their pathogenicity through inoculation tests.

4.2.2.2 Testing pathogenicity of the isolates under field conditions. A sample of 30 isolates out of 180 was tested for pathogenicity. A loopful of preserved bacteria in glycerol was withdrawn and streaked on YPGA. Typical colonies were then bulked up by sub-culturing on the same medium. Growth was removed on a sterile spatula, suspended in sterile distilled water, and diluted to a faintly visibly turbid suspension and taken to the field. One mL of bacterial suspension was injected into each of two potted tissue cultured banana plantlets via the leaf petiole using a syringe. A control plant was injected with sterile distilled water by the same technique. Plants were incubated in an isolated farmer's field and symptom development is being recorded. This experiment is in progress.

4.2.2.3 Development of pathogenicity tests to confirm identity of* Xcm *under laboratory conditions. In the absence of any suitable contained facilities (glasshouse or screenhouse) that could be used for *in vivo* work with *Xcm* at the time of these studies, the only option was to carry out pathogenicity tests in the field within an area already affected by the disease, as described in the previous paragraph. This was both time consuming and difficult to control so attempts were made to develop relatively rapid, laboratory-based pathogenicity tests that can be used to verify the identity of isolates suspected to be *Xcm* under controlled conditions. These included inoculating isolates of *Xcm* into weaned banana plantlets and into detached banana plant parts. Two sets of experiments were carried out to follow up some earlier work on detached plant parts done in December 2004, and to test the use of tissue cultured banana plantlets that could be maintained under laboratory conditions. The first set of experiments is described in Appendix 1; from this, the most promising treatments were selected for a second set of experiments that is described below.

Eight weaned plantlets of FHIA17 in their pots were brought to the lab, placed in perforated plastic bags and kept in half-Petri dishes on the window ledge in the lab, and kept well watered. They were inoculated with *Xcm* using a syringe and needle, by two methods; petiole inoculation and "stem" inoculation. Sterile water injected in petiole and "stem" served as controls. Two plants were used for each treatment. For petiole inoculation, a syringe was inserted into the petiole of the youngest fully emerged leaf and a droplet of cloudy inoculum applied. For "stem" inoculation, the syringe was inserted into the bundle of leaf sheaths at the base of the petioles, and a droplet of cloudy inoculum applied.

In another investigation involving inoculation of detached plant parts, matooke fruits and cut sections of the pseudostem core of a recently flowered plant were brought to the laboratory. Six sections of the pseudostem core, each approx. 10-cm-long, were placed on end, each in a plastic bowl containing wet paper towel to maintain humid conditions. They were inoculated by applying 5-6 droplets of cloudy inoculum to the freshly cut transverse surface of each section. Similar inoculations were attempted on sections of "exposed" peduncle from the fruit bunch itself, which were wrapped in perforated plastic bags. They were all incubated at room temperature, which varied from 20-28^{o}C. For fruits, individual fingers were removed from the bunch and left overnight to allow latex to seal off the cut ends. They were then inoculated as follows. At the distal end, the dried remains of the female flower parts were broken off, a droplet of cloudy inoculum applied, and the fruit pierced several times with a needle passed through the droplet. At the proximal end, the sealed-off surface was removed; droplets of cloudy inoculum were applied to the freshly opened surface and the fruit pierced several times as above. Fruits were wrapped in perforated polybags and left to incubate in plastic bowls placed on shelves in the lab.

It was noted that on average, plantlets inoculated via the stem developed symptoms after 12 days while those inoculated via the petiole showed wilt symptoms after 16 days. In the investigation of the inoculation of detached parts, cut ends of pseudostem core sections showed yellow ooze after 21 days while its control did not. These sections were however beginning to rot. No differences were noted between treatments and controls of "exposed" peduncle sections. These all turned brown at the cut ends and rotted away. However, ooze from internal peduncle sections was cultured on YPGA and it gave growth characteristic of *Xcm*. The fruits showed no external and internal symptoms when examined 10 days after inoculation, and they shortly started rotting.

The various pathogenicity tests suggest that it is relatively inexpensive to inoculate detached pseudostem core sections as compared to inoculation of plantlets but the sections may only be providing a growth substrate and may not discriminate between *Xcm* and other secondary invading or saprophytic bacteria. A range of other contaminants needs to be cultured on these sections to confirm specificity. Otherwise inoculation of plantlets is more confirmatory of *Xcm* identity as of now.

4.3 Field level inoculation and transmission studies

Although this output was included in the log-frame of the project document, the activities could not be fully covered during phase 1 of the project that concluded in 2005. Completion of these activities was not mandatory as per the text of the PMF. More emphasis was placed on other outputs, based on which activities under this output were carried out. The time frame of the project was too short to complete these activities. Nevertheless, useful information was obtained about the role of insects in transmitting *Xcm* under field conditions.

4.3.1 Demonstration of field insect transmission of Xcm

This experiment was established in Luwero district in July/August and in Mpigi district in September/October. In each district, two banana fields were selected using the following criteria: a) presence of BBW disease, b) size of the field (sufficient to give 10 plants per treatment), c) no de-budding in the field, d) availability of the cultivar *Kayinja*, and d) willingness of the farmer to cooperate in the experiment. In each of the selected fields, newly emerged inflorescence were identified and assigned to different treatments.

Treatments for the experiment at Luwero included: 1. Inflorescence covered with gunny bags (woven plastic sacks) into which insects were released at different development stages of the inflorescence (i.e after 1, 2 and 3 weeks after emergence of the floral raceme from the pseudostem); 2. Inflorescence bagged but no insect released; and 3. Inflorescence not bagged (control). Fifty individuals of the stingless bee *P. denoiti* collected from infected male flowers in the same field were released in each bag (Treatment 1). Prior to release, insects were tested for survival (by placing 30 insects in bags and observing survival of three insects per day for six days) in gunny bags. Samples of insects for release in bags were taken to the laboratory to determine the proportion of insects carrying *Xcm*. The treatments for the experiments in Mpigi were similar to Luwero except that five insect species (two species of stingless bees, Drosophilidae, Chloropidae and the honey bee) were released, each constituting a treatment. More than 50 insects for stingless bees *P. denoiti*, Drosophilids and Chloropids and 5-10 of honey bees and unidentified stingless bee were released in each bag. Release of insects in bags at Mpigi started two weeks after emergence of the floral raceme from the pseudostem and was repeated every week for six weeks. Plants were observed weekly to determine the stage of infection, frequency of infection, duration of incubation period and progression of symptoms. In both Luwero and Mpigi, each treatment consisted of 10 plants.

In Luwero, 20% stingless bees survived in gunny bags up to 6 days. *Xcm* was found on 23% insect samples that were released in bags. All control plants were infected by *Xcm*. In spite of the low survival of stingless bees, 20% bagged plants with released *P. denoiti* showed symptoms (Table 11). All the bagged plants without released insects were disease-free.

Table 11: Banana *Xanthomonas* wilt incidence in inflorescence covered with gunny bags into which various insects contaminated with *Xanthomonas campestris* pv. *musacearum* were released

Location	Treatment	Diseased plants (%)
Luwero[a]	Control (no bagging)	100.0
	Bagged, no insect release	0.0
	Bagged, insect release week 1	0.0
	Bagged, insect release week 2	0.0
	Bagged, insect release week 3	20.0
Mpigi[b]	Control (no bagging)	100.0
	Bagged, no insect release	0.0
	Stingless bee (*P. denoiti*)	0.0
	Stingless bee (Unidentified species)	0.0
	Grass flies (Chloropidae)	10.0
	Honey bee (*A. melifera*)	0.0
	Fruit flies (Drosophilidae)	0.0

[a] Only the stingless bee *P. denoiti* were released in bags 1, 2, and 3 weeks after inflorescences were covered with bags.
[b] Insects released two weeks after emergence of the floral raceme from the pseudostem and repeated every week for six weeks.

In Mpigi, 10% of the bagged plants in which grass flies (Family Chloropidae) were released showed BXW symptoms (Table 11). None of the bagged plants with other insects released in them showed disease symptoms. All control (not bagged) plants were diseased. The experiment in Mpigi is continuing.

In both experiments, bagging affected the behaviour and survival of the insects as shown by 80% mortality of *P. denoiti* in bags. Nevertheless, these results demonstrated that insects are involved in the transmission of *Xcm*. However, further work is required to reinforce these findings. The study indicates that practices that exclude insects from the male flower (such as bagging and debudding) can be used to manage BXW.

5. CONTRIBUTION OF OUTPUTS TO DEVELOPMENTAL IMPACT

There is incontrovertible evidence that rapid development of the BXW epidemic in Uganda has been mediated by infection via male banana flower buds. Insects have been postulated as the most likely disease vectors, and results from this project provide the first experimental evidence for the insect species involved and the likely modes of transmission. These are key findings for understanding the epidemiology of the disease and for improving strategies for its control. The results provide a scientific rationale to reinforce the campaign to persuade farmers and communities to de-bud their banana male flower buds, as well as some new information to inform them about the developmental stage at which this needs to be done. In fact, several farmers around our bagging field experiment have started debudding and their farms are being rejuvenated.

A spin-off from this work has been the investigation of bacterial distribution within diseased plants in the early stages of invasion following inflorescence infection, in collaboration with debudding trials managed by INIBAP with NARO. This work has provided experimental

evidence that there is probably a short period after the appearance of visible inflorescence symptoms before bacteria migrate as far as the base of the pseudostem. **During this period farmers can cut down the infected plant and successfully prevent systemic infection of the mat.** Although this is already a common and popular farmer practice, farmers need information about the importance of doing this early and the futility of doing it too late!

These findings give added urgency to the need for follow up activities, particularly:

- To sensitise more farmers about how, why and when to debud their bananas in order to prevent disease transmission via the male flowers
- To carry out detailed studies on the epidemiology of insect transmission of *Xcm* (spatial and temporal aspects, seasonality, importance of infection and acquisition sites etc.)
- To compare and understand the frequency and importance of insect and other modes of transmission of *Xcm* especially in different varieties, contrasting locations and cropping systems, and at different stages of the epidemic

We cannot overstress the importance of comparative studies on mechanical infection via soil and pruning wounds, and specific ways to prevent this. These are the most important modes of spread in East African Highland banana types and this is where the greatest need for practical research and control trials now remain. From both epidemiological observations and morphological considerations, spread via male flower buds is far more common, and is likely to remain a far greater problem, in ABB types and the AAB type *Sukari Ndiizi* that have become widely planted in East Africa over the past 50-60 years. Effectively, early male bud removal solves the *technical* problem of preventing spread of infection in these types. However, EAH and other AAA types grown in Uganda are far less commonly infected by this route. Other means, notably mechanical infection, via contaminated tools during pruning or cultivation, are far more important. This area is clearly critical for the future of matooke production, and hence food security, in Uganda and requires urgent and systematic investigation.

6. BIOMETRICIAN SIGNATURE

I confirm that the biometric issues have been adequately addressed in the Final Technical Report:

Signature: Philip E. Ragama
Name (typed): Philip E. Ragama
Position: Biometrician, IITA-Uganda
Date:

Appendix 1

Summary of bacteriological work done by Simon Eden-Green during during visits to KARI, ongoing observations and suggested follow up

1. Visit 12 April – 4 May 2005.

Objectives:

The main objectives of experimental work during this visit were:

1. To improve selective media for the isolation and differentiation of *Xcm* from plants, insects and the environment.

2. To make recommendations and propose draft protocols for the purification, storage and identification of *Xcm*.

This visit coincided with a visit by Dr Maina Mwangi from IITA, who came with similar objectives. We also became aware at the start of our visits that Dr Leena Tripathi (IITA Banana Biotechnology project) had carried out quite extensive preliminary work in this area, so we agreed to share the work programme broadly as follows:

(a) Carry out tests on a range of previously published selective media for various species and pathovars of *X. campestris*, and examine the sensitivity of *Xcm* and selected "problem" contaminants to some of the selective agents (mainly antibiotics) used in these media and selected from technical literature (mainly MM).

(b) Identify and test likely growth substrates utilised by *Xcm* and on which *Xcm* may have comparative advantage, either because compounds are not utilised by most other contaminating species or *Xcm* shows preferential growth or unique characteristics (mainly SE-G).

(c) Carry out further investigation of possible *in vitro* pathogenicity tests that could be used to confirm the identity of *Xcm* under laboratory conditions (SE-G).

(d) Prepare draft protocols for general bacteriological procedures, in discussion with others (SE-G to draft, all to contribute).

Work carried out

METHODS

A. Tests on growth possible substrates selective for *Xcm*.

A preliminary selection of growth substrates utilised by *Xcm* was made from examination of Biolog data provided by CABI. The Biolog system consists of a series of standard metabolic tests (95 tests + control) performed in microtitre plates that have been pre-loaded with various substrates, selected for ability to differentiate different groups and species of bacteria. Biolog was devised mainly with bacteria of medical and environmental importance in mind, but has

some value for profiling plant pathogenic bacteria. The test uses a redox indicator (triphenyltetrazolium chloride, TZC) to detect the catabolism of various carbohydrates, amino acids, organic acids and other compounds. Compounds that are catabolised may or may not be used as sole sources of carbon and energy; however, this is frequently the case, so the Biolog results provide a guide to selection of substrates that may be preferentially used by *Xcm*.

Results of six Biolog tests, on four isolates of *Xcm* (i.e. including two duplicate tests) were available from CABI, together with the "generic" profiles for *X. campestris* and *X. arboricola*. From these, about 22 compounds were identified that were utilised by all isolates of *Xcm* in at least one of the duplicate tests. However, only about half of these were available at Kawanda (including some ordered specifically for this visit). Several of these compounds (such as glucose, sucrose and other common sugars) are unlikely to have any differential value but were included in the tests as positive controls. The compounds tested were:

Substrate	**Code**
cis-aconitinic acid (neutralised with KOH, so as potassium salt)	ACO
Cellobiose	CEL
L cysteine HCl*	CYS
D galactose	GAL
D glucose	GLU
L glutamic acid (neutralised with KOH, so as potassium salt)*	GLT
acetate (as potassium salt)	KAC
D mannose	MAN
L methionine*	MET
Sucrose	SUC
Xylose	XYL
Control (water)	H20

All substrates were prepared in deionised water at 1% w/v concentration (i.e. 0.1g/10ml). Bromothymol blue (30µg/ml final concentration) was included in water used to make stock solutions and in all basal media, and the pH was adjusted to about 7.4 (green/blue colour) with 1N KOH (only necessary for aconitinic acid). Most substrates were sterilised by autoclaving except for those marked "*" which were filter sterilised.

Experiment 1, inoculated 27 April:

Plates were prepared of:

Ayers, Rupp & Johnson minimal agar medium without yeast extract (ARJ –YE)
Ayers, Rupp & Johnson minimal agar medium with 0.05% yeast extract (ARJ +YE)
Yeast extract peptone agar (YPA; 3g YE, 5g peptone, 12g Difco agar per litre)

Plates were marked on the base so as to divide them into six sectors. Each sector was labelled with a substrate code, as indicated above, the intention being to "spot" all 12 substrates onto two plates of each medium. Four isolates were tested in duplicate so eight plates of each medium were labelled with each of six combinations of substrates.

Plates were inoculated by spreading with 0.1ml of a freshly prepared, faintly turbid suspension of the following bacteria, taken from plates recently prepared by others:

Xcm "stock" culture, obtained by Maina from L Tripathi (= XCM)
Recent *Xcm* isolate from Fred Ssekiwoko 011WKS05 (= WKS)
Contaminant "018" from Maina (= 018)
Contaminant "n" from Maina (= N)

Following inoculation, the plates were allowed to dry in the laminar flow bench and 20µl of each substrate was spotted onto the appropriate pre-labelled position on each plate. Plates were placed in plastic bags and incubated at 25°C.

Experiment 2, inoculated 30 April:

Carried out exactly as above except that only ARJ+YE was used; holes (about 7mm diameter) were cut in the agar surface before inoculation, to enable 60 µl of each substrate to be applied. Inoculum was prepared by suspending growth from plates inoculated on 27 April.

B. Test on antimicrobials.

Only one experiment was set up as this area was largely covered by Maina and had already been explored by Leena. The objectives were to test a few of the antimicrobials that had not been extensively tested by others using gradients of concentration, to allow direct comparison of the sensitivities of *Xcm* and the two contaminants tested above.

Experiment 3, inoculated 30 April:

A basal medium was prepared consisting of yeast extract (0.5% w/v) tryptone (0.5% w/v) agar (1.2% Difco) + 1% galactose + 25µg/ml cephalexin (YTA+ gal). Galactose was autoclaved separately as a 10% solution and added to 9x the volume of autoclaved, molten YPA. Cephalexin was prepared as a 100x stock solution in water, filter sterilised and added to 100x the volume of molten YTA + gal. The sterilised medium was kept molten in a water bath at approx. 55°C. Where possible, antimicrobials were prepared at 100x final concentration, in water or 96% ethanol. Aqueous solutions were filter sterilised, and ethanolic solutions were regarded as self-sterile. Two compounds could not be dissolved in either solvent so were carefully weighed into sterile universal bottles and suspended in 0.5ml of 96% ethanol to sterilise them. 150µl of each 100x antimicrobial solution was then pipetted into separate sterile universal bottles (duplicate bottles for each compound). Approximately 15ml (measured by eye) of molten agar base was added to each bottle, swirled to mix, and immediately poured into a 90mm plastic Petri dish. Each dish was tilted slightly to create an agar slant and allowed to set. When set, a further layer of approx. 15ml basal medium was poured onto the slants which were then allowed to set in a horizontal position, thus creating a gradient of antimicrobial concentration across the plate, with the direction of each gradient being marked by an arrow. Plates were allowed to dry and were then inoculated with single, parallel, streaks of each test organism across the gradients (inoculum the same as Expt. 2). Plates were incubated right-side up. Compounds tested (and solubilities) were:

Compound & code	How prepared	Final concn. in 15ml agar
"cetrimide"* CET	100mg/5ml warm water	Add 150µl → 200µg/ml
Ciprofloxacin CIP	1.5mg + 0.5ml ethanol	100µg/ml
Cephalexin	12.5mg in 5ml water	5ml in 500ml → 25µg/ml (in basal medium only)
Cephalothin CLN	50mg in 5ml water	Add 150µl → 100µg/ml
Colistin COL	25mg in 5ml water	Add 150µl → 50µg/ml
Erythromycin ERY	50mg in 5ml ethanol	Add 150µl → 100µg/ml
Nalidixic acid NAL	50mg in 5ml ethanol, dissolved and neutralised with 4-5 drops of 1N KOH	Add 150µl → 100µg/ml
Nitrofurantoin NIT	1.5mg + 0.5ml ethanol	100µg/ml
Water control C		Add 150µl

* cetrimide = cetyltrimethylammonium bromide; CTAB.

C. Pathogenicity tests.

Experiment 4, inoculated 27 April:

The objective was to develop a relatively rapid, laboratory-based test that can be used to verify the identity of isolates suspected to be *Xcm*. The following plant materials were used:

(a) Weaned plantlets of FHIA17 and Mpologoma from the screenhouse at the Resource Centre (courtesy of Priva and Martha). Approx. 2 weeks after transplanting. 10 of each. Plants were left in their polybags, returned to the lab, placed in perforated plastic bags and put in half-Petri dishes on the window ledge in the prep room and kept well watered.

(b) Parts of three mature EAH (matooke) plants:

Plant 1: Kabula. About 2 weeks after shooting, male bud just differentiated and ready for debudding.

Plant 2: Kisubi. Fruits about half-full.

Plant 3: Kabula. Fruits ready for harvest.

Whole fruit bunches and sections of mid-pseudostem (about 75 cm long) were returned to the lab. The stem sections were dissected to remove the flower stalks (peduncles) and bagged. Individual fruits were removed from the oldest bunch and left overnight to allow latex to seal off the cut ends. Immediately before inoculation, about six sections (approx. 10cm long) were cut from each plant and two of each were placed on end in three plastic boxes containing wet paper towel (humid chambers).

Inoculum consisted of suspensions of two isolates (XCM and WAK) and a sterile water control (C), as used in Expt 1, above.

Plantlets were inoculated with a syringe and 23 gauge needle by two methods (two reps per treatment):

Petiole inoculation (PET), by inserting the syringe into the petiole of the youngest fully-emerged leaf and applying a droplet of inoculum;

"Stem" inoculation (STEM), by inserting the same needle into the bundle of leaf sheaths at the base of the petioles, and applying a small (but variable) volume of inoculum (needle blockage was a problem). Inoculations were done by inserting the needle through the bags, which were closed at the top with rubber bands.

Peduncle sections were inoculated by applying 5-6 droplets of inoculum to the freshly cut transverse surface of each peduncle section (the droplets adsorbed very quickly). Similar inoculations were attempted on sections of "exposed" peduncle from the fruit bunch itself, which were wrapped up in perforated plastic bags.

After some experimentation, fruits were inoculated at both ends as follows. At the distal end, the dried remains of the female flower parts was broken off, a droplet of inoculum applied, and the fruit pierced several times with a 21 gauge needle passed through the droplet, which in most cases adsorbed quickly. At the proximal end, the sealed-off surface was removed, droplets of inoculum applied to the freshly cut surface and the fruit pierced several times as above. Fruits were wrapped in perforated polybags and left to incubate in plastic bowls placed on shelves in the prep room.

For the first two days after inoculation, the prep room was airconditioned and the temperature in the low 20s. Airconditioning was off on 30 April and 1 May and temperatures higher, particularly on 30 April when autoclaves were functioning. A/C was on again on 2 May onwards.

RESULTS (as at 2 May; with postscript on further observations by KARI staff)

Experiment 1, inoculated 27 April (now 5 days after inoculation):

All XCM, WKS and N plates show contamination of some of the substrates, notably GAL, GLU, KAC, MET, SUC, XYL and H20 (origin unknown; could be non-sterile tips or possibly introduced whilst applying droplets to the pre-inoculated plates). Hence interpretation is limited.

Good, uniform, growth of all isolates on YPA medium (which had become alkaline) and no signs of stimulation of growth around any substrate.

All isolates showed good growth on ARJ+YE but growth of XCM and WAK was less dense than on YPA. Medium slightly alkaline and no clear signs of stimulation around any substrate. As was to be expected, all isolates showed only slight growth on ARJ-YE; no signs of stimulation around any substrate but isolate N appeared to be inhibited by ACO.

The observations should be maintained for about 10 days but it seems unlikely that any clear differences will emerge, probably because the amount of growth substrate that could be applied to the plates was too small. Contamination will also limit the value of any conclusions. Further work – see Expt. 2

Experiment 2, inoculated 30 April (now 2 days after inoculation):

This experiment was similar to Expt 1, but only ARJ+YE was used and substrates were applied as 60 µl volumes to wells cut in the pre-inoculated agar. At the time of recording, there was obvious growth of N and 018, with no signs of stimulation around any of the substrate wells, and slight growth of WKS, again with no signs of stimulation. Isolate XCM showed slight growth only around the CYS substrate well on both plates, suggesting that CYS could be preferentially utilised.

Continue observations for about 10 days and record any differences between treatments. However, I think it is unlikely that this technique will work unless the concentration of substrates can be increased. It would be possible to prepare 10% or 20% stock solutions of most of the sugars but other compounds may not be soluble at these concentrations. An alternative technique is to incorporate the substrates directly into the growth medium (usually recommended at 1% or 0.1%) and streak with bacterial suspension, which preferably should be well washed to remove any residual nutrients from the medium used to prepare inoculum, or have been prepared on the basal medium without added carbon source (e.g. ARJ+YE).

From the limited observations so far, it would be worth testing aconitinate, cysteine, methionine and potassium acetate and any other organic and amino acids that may be available. To counteract increase in pH on these substrates (presumably the effects of deamination), additional buffering may be necessary (increase concentration of $NH_4H_2PO_4$). Alternatively, include a low concentration of a carbohydrate (preferably one preferentially utilised by *Xcm*), from which *Xcm* acid production should counteract production of alkali.

It seems clear that a key to reducing overgrowth by contaminating bacteria is to keep the carbohydrate concentration in the growth medium as low as possible, without reducing the rate of growth of *Xcm*. In previous experiments (2004) colonies of *Xcm* appeared quickly on 1% tryptone agar, even without added yeast extract or carbohydrate, so this might be a better starting point for developing a selective medium than YPA. Suggest further experiments to establish optimum concentrations (as simply measured by speed of appearance and rate of growth in colony diameter) of tryptone with and without added yeast extract, then test for optimum concentrations of additional growth substrates that have been selected from carbon source utilisation tests on a minimal salts medium such as ARJ.

Experiment 3, inoculated 30 April (now 2 days after inoculation):

No growth any isolate on CIP and NAL plates.
On NIT plates, 018 shows reduced growth (small colonies) over approximately half of gradient; WAK also shows slight growth over same part of plate.
On COL plates, 018 has grown abundantly over >half of the gradient, overgrowing the other isolates.
On all other plates, 018 has unfortunately grown so abundantly that it is overgrowing all the other isolates, although growth of XCM seen on one of the 2 control plates.

Unfortunately, it appears that inclusion of a high concentration of galactose in the basal medium has encouraged abundant growth and slime production of isolate 018, so further observations will only be possible on a few of the gradient plates; these should be continued for up to 10 days. It would be worth repeating this and similar tests using low sugar, or sugar-free, medium, especially to see of CIP, NAL, NIT and COL show any differential

activity. However, all of these antibiotics are known to inhibit growth of Gram –ve bacteria so it is unlikely that any will be useful in a selective medium.

It was notable that growth of 018 and N on the sugar-free YPA used in Expt 1 was slime-free and much less "rampant" than in this experiment.

Experiment 4, inoculated 27 April (now 5 days after inoculation):

No symptoms seen in any of the test plants, some of which are now becoming cramped within the bags. "Exposed" peduncle sections are going mouldy at cut ends and will have to be discarded. Cut ends of internal peduncle sections are dry and slightly brown, no differences between treatments and controls. Fruits show no external symptoms apart from youngest fruits (plant 1), all of which (including controls) are developing necrotic spots and are unlikely to yield any results.

Maintain observations for up to three weeks, or as long as possible before the plants outgrow the plastic bags or the detached peduncles and fruits become rotten. Suggest cut open one of the two fruits used for each treatment at about 10 days after inoculation, to see if there are any internal symptoms.

If any plantlets or detached plant samples develop symptoms then it will be important to re-isolate *Xcm* immediately, in order to prove Koch's postulates.

Postscript – Further observations on Experiment 4 by Fred Ssekiwoko and colleagues:

Plants inoculated by "stem" inoculation developed symptoms between 6 – 9 May (9-12 days after inoculation) and those inoculated by petiole inoculation between 16-18 May (19-21 days after inoculation). Two FHIA17 control plants (one stem and one petiole inoculation) also developed symptoms; these may have been inadvertently inoculated with bacteria or possibly could have become contaminated by overflow of water from one pot to another when watering. No re-isolations were attempted. Internal peduncle sections produced ooze after 19 May (22 days after inoculation). No further observations were reported from other experiments started during my visit.

2. Visit 29 June – 15 July 2005.

METHODS

Two experiments were carried out.

Experiment 1: Further carbon source utilisation tests

Plates were prepared of: yeast extract tryptone agar (YTA; 5g YE, 5g tryptone, 13g "Indian" agar per litre); Ayers, Rupp & Johnson (ARJ) minimal agar medium supplemented with one-tenth strength YT (0.5g YE, 0.5g tryptone per litre). Carbon sources selected from availability in the laboratory (aconitinic acid, cellobiose, ferric citrate, hippuric acid, glucose, methionine, potassium acetate, trehalose, xylose) were prepared at 10x final strength (i.e. 10% w/v) solutions in water. Bromothymol blue (30µg/ml) was included as pH indicator in all solutions and the organic acids were dissolved in a small volume of 10N KOH and brought to neutrality before autoclaving. Plates were prepared in duplicate with a gradient of each carbon source by carefully mixing in each plate 1.2ml of each 10x strength carbon source with about 12ml of cooled, molten agar then tilting the plate to allow the agar to set as a slope. Directions of the slopes were marked on the bottom of the plate, and an upper layer of agar without carbon source was added and allowed to set in a horizontal position so as to form a gradient of concentration of each carbon source. Inoculum was prepared in sterile distilled water as a faintly turbid suspension of a recent isolate of *Xcm* (from Bushenyi) and loopfuls streaked across the gradients. Plates were incubated at 25° as usual and observed for colony size and morphology.

Experiment 2: Growth of Xcm on media with low water activity (high osmotic pressure)

Media were prepared containing different strengths of sorbitol (MW=182) at 0%, 0.5%, 5%, 10% &15%) and double the same strengths of sucrose (MW= 342) 0%, 1%, 10%, 20% & 30%) in yeast tryptone agar. A low concentration of sucrose (0.5%) was included in all of the sorbitol media to provide a carbon source known to be utilised by *Xcm*. All media were made up by adjusting the final volumes to achieve the correct %(w/v) of solution and sterilised by autoclaving. Separate plates were inoculated by streaking with suspensions of recent isolates from Nakifuma and Bushenyi, incubated at 25° as usual and observed for colony size and morphology.

RESULTS

Experiment 1:

Growth (maximum colony size and appearance) of an isolate of *Xcm* (from Bushenyi) on ARJ medium plus gradients of concentration of different substrates, after 3 and 6 days incubation at 25°C

Substrate	Colony appearance at 3 days	Colony appearance at 6 days
Water	>1mm, white	>2mm, pale yellow, raised, non-mucoid
Acetate[1]	Growth medium acid and no growth	Growth medium acid and no growth
Aconitinate[1]	1mm, sparse at high concentration	>4mm, good growth across gradient, yellow, raised, non-mucoid
Cellobiose	1-2mm, good growth	5-6mm, yellow, mucoid
Citrate[2]	Growth medium acid, no growth	Growth medium acid, no growth
Glucose	<1mm, slightly larger at higher concentration	6-7mm, yellow, mucoid
Hippurate[1]	<1mm at low concentration, inhibited at approx. half of gradient	2-3mm at low concentration, no growth at high concentration
Methionine	Minute colonies, some inhibition at highest concentration	Minute pale yellow colonies, some inhibition at highest concentration
Trehalose	2mm at highest concentration	6-7mm, yellow, mucoid
Xylose	<1mm at lowest concentration, inhibited at higher concentrations	4-5mm at lower, smaller at higher concentrations, yellow, mucoid

[1] as potassium salt
[2] as ferric salt

Growth (maximum colony size and appearance) of an isolate of *Xcm* (from Bushenyi) on YTA medium plus gradients of concentration of different substrates, after 3 and 6 days incubation at 25°C

Substrate	Colony appearance at 3 days	Colony appearance at 6 days
Water	1-2mm, white	4-5mm, yellow, raised, slightly mucoid
Acetate[1]	Growth medium acid and no growth	Growth medium acid and no growth
Aconitinate[1]	1-2mm, inhibited at high concentration	4-5mm, slightly inhibited at highest concentration, yellow, non-mucoid
Cellobiose	1-2mm, good growth	7mm, yellow, mucoid
Citrate[2]	Growth medium acid and no growth	Growth medium acid and no growth
Glucose	1mm	5mm, yellow, mucoid
Hippurate[1]	1mm at low concentration, inhibited at approx. half of gradient	4-5mm at low concentration, no growth high concentration on one plate, yellow
Methionine	<1mm, size reduction across plate and inhibited at highest concentration	2-3mm, yellow, non-mucoid, inhibited at higher concentrations
Trehalose	>2mm at highest concentration	9mm, yellow, mucoid, slightly larger at higher concentrations
Xylose	1-2mm at lowest concentration, smaller at higher concentrations	5-6mm across plates, yellow, mucoid

[1] as potassium salt
[2] as ferric salt

Conclusions: Colonies appeared quickest, and grew largest, on media containing sugars, especially the disaccharides cellobiose and trehalose (would be interesting to compare with sucrose, but not included here). Xylose, aconitinate and hippurate appear to be utilised but may be inhibitory at higher concentrations. These might be useful as differential growth substrates but unfortunately do not encourage rapid growth of *Xcm*. YTA alone gave good growth of *Xcm* and reduces the problem of excessive slime production .

Experiment 2:

Growth (maximum colony size and appearance) of two isolates of *Xcm* (from Bushenyi and Nakifuma) on YTA medium plus different concentrations (%w/v) of sucrose after 4 and 8 days incubation at 25°C

%	Bushenyi isolate		Nakifuma isolate	
	4 days	8 days	4 days	8 days
0.0	<3mm	6-7mm, non-mucoid	>3mm	4-5mm, non-mucoid
1.0	>3mm	9-10mm, slimy	<3mm	9-10mm, slimy
10	4mm	10-11mm, v. slimy	3-4mm	9-10mm, v. slimy
20	2-3mm	5-6mm, watery, sparse on secondary	Slight watery growth on primary	6-7mm, mucoid, sparse on secondary
30	No growth	No growth	No growth	No growth

Growth (maximum colony size and appearance) of two isolates of *Xcm* (from Bushenyi and Nakifuma) on YTA + 0.5% glucose medium, plus different concentrations (%w/v) of sorbitol after 4 and 8 days incubation at 25°C

%	Bushenyi isolate		Nakifuma isolate	
	4 days	8 days	4 days	8 days
0.0	3mm	8mm, mucoid	3mm	7-8mm, mucoid
0.5	3mm	7-8mm, mucoid	3mm	8-9mm, mucoid
5.0	3-4mm	7-9mm, mucoid	<3mm	5-6mm, mucoid
10	≤2mm,	7mm, mucoid	<2mm	2-4mm, mucoid, sparse on secondary
15	No growth	No growth	No growth	No growth

Conclusions: Both osmotica gave similar results at approximately equivalent molalities. Growth of *Xcm* was slightly inhibited at 10% and 20%, and completely inhibited at 15% and 30%, of sorbitol and sucrose, respectively. It seems unlikely that a water activity equivalent to that of 10% sorbitol or 20% sucrose would inhibit the growth of other bacteria but it might be worth testing this using a metabolically inert substrate (possibly such as inositol) that would not encourage the growth of other organisms.

Appendix 2

Suggested standard bacteriological procedures for work with *Xcm*

General precautions for handling *Xcm*:

Although the risk of *Xcm* escaping from the lab is probably small, it should be treated as a dangerous quarantine pathogen. Researchers are less likely to be blamed for any new outbreak that may occur on station if they can show that they have taken robust precautions to contain it within the lab. The following guidelines are suggested:

Designated persons and training: Only "designated persons" should be allowed to handle or work with *Xcm*. "Designated persons" should be those who

- have proven to the head of the National Banana Programme (or someone delegated by him or her) that they have appropriate technical competence
- are aware of the risks involved in working with this bacterium
- will be adequately supervised

These guidelines should apply to all students, new members of staff and visiting workers. Appropriate training (including provision of these guidelines) should be given to all persons who wish to become designated persons.

Work areas: Detailed and specific guidelines should be developed to define

- designated areas where *Xcm* may be handled and stored
- which types of activities should be allowedin those areas
- which precautions need to be taken in those areas

For instance, all work with unconfined bacteria (isolations, inoculations, examination of slides or open Petri plates) should be carried out under conditions of highest containment, such as within the bacteriology lab or at least within a confined area (such as a laminar flow hood, carefully disinfected after use) in one of the labs within the biotechnology building (which has itself been designed as a biological containment area). Other activities, such as preparation of culture media, storage of chemicals, equipment, notebooks etc.) should be kept out of high-containment areas so as to reduce the risk of bacterial escape through human activity.

Cleanliness and tidiness: Maintaining clean and tidy conditions in high-containment areas will not only reduce the risks of escape but will also help to reduce contamination of growth media. It follows that there must be adequate storage facilities for chemicals, small apparatus, notebooks, etc. and preferably these should not routinely be kept in the main area used for isolations and inoculations. Personal hygiene is also important: lab coats should be worn and not removed from the lab, except for washing using hot water; hands should be washed with antibacterial cleanser before leaving the lab; any spillages that contaminate clothing or footwear must be decontaminated on the spot by washing with copious quantities of ethanol or dilute hypochlorite.

Decontamination and disposal of cultures, plant materials and consumables: Procedures must be agreed upon, rigorously adopted and maintained. Suggestions include

- Small apparatus (scalpels, knives, scissors, forceps, etc.): rinse or swab well with >70% ethanol, followed by flaming where safe and practicable.
- Discarded cultures (Petri dishes, bottles, vials, slides, etc.): for disposable containers, place in autoclavable plastic bags, seal with tape or rubber band and autoclave. Treat non-disposables (glass bottles, plates, etc.) in same way, or alternatively open containers and immerse in fresh hypochlorite (10% Jik) for at least 30 minutes before washing up.
- Contaminated plant materials and soil: Place and seal in autoclavable plastic bags and autoclave as soon as possible. If plant remains are too large to autoclave then place them in a large plastic bag or bin (with lid) and dispose them safely by burning, or burying in an area already affected by disease.
- DO NOT LEAVE CONTAMINATED MATERIALS LYING AROUND, even after autoclaving. These will attract insects such as ants and flies, giving rise not only to increased risks of contamination but also risks of spread of BBW. Contaminated materials should be sterilised the same day and the residues then removed from the lab. If insects become a problem, or are likely to interfere with an experiment or equipment, use an insecticide spray designed to treat surfaces.

Other safety precautions and care and maintenance of equipment

Common hazards and laboratory safety: Everyone using the lab should receive some basic training in laboratory safety, including hazards of common laboratory chemicals, spillages, disposable of hazardous materials, what to do in the event of accidents, etc. Every procedure involving particular risks (such as use of especially toxic reagents) should first be assessed and appropriate procedures considered and agreed upon with the head of the NBRP or someone designated by him or her as the person responsible.

Maintenance and repairs: **Everyone using the lab must accept responsibility for reporting malfunctioning equipment, lab fittings or furniture, and following through with further reports until something is done.** Even trivial problems, like water leaks, faulty switches or electrical sockets, can cause significant damage if not attended to quickly.

Stocks, storage and replenishment of consumables: Similarly, everyone must accept responsibility for ensuring that as stocks of consumables, such as paper towels, ethanol, Petri dishes, culture media ingredients, etc. are getting low then these are ordered or replaced in good time. It might be helpful to maintain a communal laboratory diary to keep a record of what is required, who has taken action and what has been done.

Disposal of rubbish and used consumables: Current procedures for disposal of laboratory rubbish, including autoclaved cultures, used pipette tips, syringes, etc., are unclear to me and should be discussed. Many of these materials are potentially hazardous and need to be disposed of safely.

Culture media, reagents and equipment

General notes on preparation and storage: It should be standard practice to maintain stocks of standard media, reagents, sterile Petri dishes, culture bottles etc. within a "clean" area (i.e.

a room that is kept tidy, regularly and thoroughly cleaned, and used only for microbiological work.) It follows that this room should not be used for general storage or as a general office or work area – other lab, office and storage space is required for these purposes. As soon as small equipment such as glass bottles or dishes have been used these should be washed up, dried (if necessary), sterilised and stored in racks or baskets in the clean room. Likewise, as soon as experiments have been finished and recorded, equipment should be removed from the clean area and washed up or disposed of.

Autoclaving procedures and precautions – some points to note:

- Sterilisation is achieved by holding samples for a minimum period of time at a minimum temperature. Typically this is 15 minutes at 121°C , but sterilisation times may need to be increased for large sample volumes. A temperature of 121°C will only be attained if all air has been expelled from the autoclave at the beginning of the run. This should be done automatically during automated autoclave cycles but manual autoclaves should be allowed to steam for a few minutes before building up pressure.
- Some plastics, such as polypropylene and Nalgene, will survive autoclaving,;others such as polyethylene and polystyrene will not. As a general rule if the plastic retains its shape in boiling water then it is safe to autoclave. Autoclavable bags, indicator tape, etc., are expensive – so ONLY use these for autoclaving, not for routine sample storage or sealing.
- Small glass bottles with screw caps (7.5ml bijou, 15ml or 30ml McCartney, 30ml Universal) can safely be autoclaved with the caps tightly closed, and this is the best way to sterilise these bottles either empty or full. Larger bottles should be autoclaved with lids slightly loosened to allow internal pressures to equilibrate with those of the autoclave. Lids must be tightened when the autoclave is opened after a run.
- Autoclaving may change the pH of some culture media. This can be adjusted by including an internal pH indicator (such as bromothymol blue) and adding drops of sterile 0.1N KOH or HCl.
- Some substances may degrade on autoclaving, or change the composition of growth media. As a general rule, carbohydrates (common sugars) are reasonably stable but amino acids, growth factors (vitamins etc.) and antibiotics may not be. The latter should be autoclaved separately or sterilised by other means.

Other sterilisation and disinfection methods (filtration, chemicals etc.):
Many compounds can be prepared as a concentrated solution (e.g. 10x, 50x or 100x working strength) and added aseptically to culture media after autoclaving or sterilised by other means. Some techniques are:

- Prepare as self-sterile solution, usually ethanol (best at 70% v/v, alternatively 96% v/v but some bacterial spores may survive). Ethanolic solutions should not be added at concentrations greater than 1:50 otherwise the concentration of ethanol may inhibit growth.
- Add a few drops of chloroform, shake solution well and store in fridge overnight. This not a well-recognised procedure but is usually effective provided that the chloroform is allowed to separate from the solution before adding solution to growth media.
- Filter using a sterile membrane filter, usually using a syringe to push the sample through a pre-sterilised disposable membrane filter holder or a filter fitted to a reusable filter holder and sterilised by autoclaving. For most purposes, 0.45µm average pore diameter (apd) filters can be used to remove contaminating

microorganisms. Filters with 0.22µm apd are usually recommended for absolute sterility but even these will allow the passage of mycoplasmas and of course bacteriophage and viruses. Apply a steady but moderate pressure to the syringe when filtering. Excessive pressure may damage the filter, resulting in loss of sterility in the sample. Much particulate matter and will need to be clarified (for instance by centrifuging) before attempting to filter again.

- *Laminar flow benches:* Laminar flow benches help to reduce contamination if used correctly, but they are not a substitute for careful aseptic technique and maintaining a clean laboratory. It should be possible to carry out most microbiological work without recourse to using a laminar flow bench, as was standard practice until the late 1960s! Current practices for using laminar flow benches need to be improved. In particular: Benches should not be used as a storage area. When work has been completed, remove all used samples, plates, glassware etc. and CLEAN UP!
- The space between the filter and the work area must be kept clear of obstructions, so that a smooth "laminar" flow of sterile air is maintained around the work being carried out. If any bottles, plates, samples etc. have to be placed within the cabinet at all, then these should be kept to the sides of the work area and not placed in front of the filter where they can obstruct the airflow.
- In time, the airflow will reduce as prefilters and final filters become blocked. Some types of prefilter can sometimes be cleaned; others (and all final filters) need to be replaced from time to time and replacements should be ordered in good time. Filters will last longer in a clean, dust-free environment.
- Do not leave the fans running when the bench is not in use or run them unnecessarily, for instance when work could be done outside the bench.

Choice of culture media: Details of culture media suitable for *Xcm* are beyond the scope of these guidelines, but some general observations may be useful.

- Get to know the growth characteristics of *Xcm* on commonly used media and under standard conditions of incubation (see identification, below).
- Be prepared to modify standard media for different purposes. For instance, it may be much easier to carry out plate counts on a medium that reduces slime production and gives smaller and better separated colonies. This can be achieved simply by leaving out the sugars (glucose, sucrose, cellobiose etc.) from media that contain peptone or tryptone. This may also reduce problems caused by overgrowth of faster growing contaminants.
- Work on selective media for *Xcm* is still in progress but is doubtful whether a fully selective medium can be achieved, at least without reducing the plating efficiency or the rate of growth.
- *Xcm* is an aerobe (i.e. requires oxygen for respiration) so Petri plates and culture bottles should not be tightly sealed during incubation. Plates are best incubated upside down in plastic bags with the openings lightly closed, to allow gas exchange but reduce drying out.

Culture techniques

General principles regarding selection and storage of samples: Avoid as much contamination as possible by keeping field samples cool and isolating them as soon after collection as possible. Do not store plant samples in the refrigerator for more than 5 days and preferably no more than 2 days. *Xcm* may survive poorly in water, especially at low

concentrations (this needs to be tested) so use a diluent such as 0.01M phosphate buffer or deionised water containing 1% growth medium.

Procedures for isolating from plant parts: Avoid material likely to be contaminated by secondary invaders, saprophytes or surface contaminants. If this is impossible, then make a 10x dilution series and try isolating from this so as to dilute out contaminants. Use selective media (if available) but include a non-selective medium as a check. When examining a new or unknown disease, use the microscope to see what is there preferably before isolating. This can give much more useful information than culturing alone.

Procedures for isolating from insects: So far as *Xcm* is concerned, the main interest will be to establish which insects are carrying bacteria and where these are being carried on or within the insect's body. Some preliminary conclusions can be drawn by using the following procedures:

- Wash insect(s) by placing and shaking them in sterile diluent (see above) containing a wetting agent such as 0.001% Tween 80 (check survival of *Xcm* first). Presence of *Xcm* in washings indicates bacteria carried on insect surface such as legs or mouthparts (but could have been regurgitated during washing).
- After washing, transfer insects to fresh diluent and pull off the head capsule from the thorax, whilst submerged in diluent. Presence of *Xcm* in diluent indicates bacteria in hemolymph of possibly alimentary canal (might be possible to differentiate by removing legs rather than head capsule).
- Place whole body or body parts in small volume of diluent and macerate them to release any internal bacteria. Several types of apparatus have been described but a simple technique is to use a miniature pestle and mortar consisting of a 20-30mm solid watchglass and a 6-8mm dia glass rod, rounded at the 'business' end. After each sample, the same piece of apparatus can be washed out with water followed by 96% ethanol, sterilised by burning off the ethanol, and then used for the next sample.

Recognition and preliminary (or presumptive) identification: Observe and record growth and colony characteristics, etc. (e.g. rate of appearance of colonies, which may be affected by temperature; colony appearance, including shape, profile, colour, consistency/slime production; appearance under stereo microscope; even smell in pure culture). For many purposes this will be sufficient for identification. However, never assume that all colonies that look like *Xcm* really are *Xcm*, or that all other colonies of *Xcm* are not *Xcm*. Variants may arise that have atypical colony morphology.

Purification: Never assume that the presence of a single colony type represents a pure culture, especially on first isolation from a natural habitat or on a selective medium. Other organisms may be present as minute colonies or mixed in the same colonies. If it is necessary to retain a subculture from the isolation, pick two or three typical colonies and subculture at least once onto non-selective medium as soon as possible by streaking carefully to obtain well-separated colonies. Repeat until only pure colonies are obtained from at least two successive cultures. Finally preserve growth preferably from only two or three typical, well-separated colonies.

Preservation and storage of cultures: When subcultures appear to be pure (i.e. a single colony type, or a consistent mixture of variants) preserve by suspending growth from one to three colonies in cryoprotective medium and freezing at -70°C (best for long-term storage)

and (where desired for short-term tests) also on yeast peptone agar slopes kept refrigerated or at room temperature (survival of *Xcm* under these two regimes needs to be checked and compared). It is best to assign a simple, unambiguous reference code to each purified culture at this stage and ensure that full details are recorded both on paper and as a back-up electronic copy. Agar slopes prepared in 7.5ml bijou bottles or in 1.5ml screw-capped eppendorf vials provide a convenient way of storing cultures and are also suitable for sending samples to others.

Identification of *Xcm* and other bacteria

Presumptive identification based on colony characteristics on a standard medium such as YPA, incubated and observed under standard conditions, will be sufficiently accurate to identify *Xcm* isolated from diseased banana tissues for most purposes; however, it should still be backed up from time to time with definitive identification of a small, random, sample of isolates. Isolates from other sources are more likely to include other xanthomonads or other species with similar colony characteristics to *Xcm* so as a minimum, more definitive idetification tests should be carried out on a systematic sample (perhaps 5% or 10%) of all isolates from important experimental work. All determinative tests should include both positive and negative controls, as well as non-inoculated test media, and it may be convenient to wait until several isolates can be tested together in batches. When reviving cultures from storage for determinative tests, first subculture to check for purity and uniformity, reselecting typical colony types if necessary, and preferably carry out tests on young (usually 1-3 day old) subcultures.

Definitive identification of bacteria *in vitro* to species or host-specific strain is difficult. Current taxonomic schemes are constantly evolving and rely extensively on comparison of biochemical composition, such as cell wall fatty acid methyl esterase (FAME) analysis, or genome characteristics (such as pulsed-field electrophoresis of bacterial DNA, nucleic acid probes, specific PCR products, restriction profiles, sequencing of ribosomal DNA or other genes) amongst large collections of isolates. As a result, definitive identifications are becoming the preserve of the few laboratories that are equipped with the necessary facilities and access to culture collections. However, presumptive identifications to genus, and sometimes species or strain, can usually be made from a far simpler range of cultural characteristics or metabolic tests, such as in schemes proposed in Fahy & Persley (1983) and Schaad et al. (2000). Key characteristics for *Xanthomonas* that can be tested at KARI are: Gram negative rods, strictly aerobic growth (but may be slow to produce acid from glucose), and production of methanol-soluble yellow xanthomonadin pigment with a characteristic absorption spectrum. Molecular properties can also be used and it is possible that antibodies, currently under development, will also have sufficient specificity to be useful for identification to pathovar, or at least species, level.

Host tests: Pathovars of *Xanthomonas* are defined by their ability to cause disease in specific plant hosts. Therefore, provided an isolate conforms to the general characteristics of the genus, confirmation of pathogenicity to banana will provide a definitive identification of *Xcm*. Given the ready availability of banana plants at Kawanda, this is the most practical approach to definitive identification and should be a one of the tools for routine diagnosis. It is not always necessary to use the same host from which a suspect isolate was originally derived (homologous host). Alternative hosts, often within the same genus or family, may be more convenient or readily available, and detached plant parts (such as leaves, pods or storage organs) can sometimes be used and maintained under laboratory conditions. As a

general rule, younger plant tissues are more susceptible, or will show symptoms more quickly than older ones, and the types of plant tissue to be tested, and their route of inoculation should reflect those seen or suspected in the original disease.

Because of perceived risks of introducing *Xcm* on station, pathogenicity testing has so far had to be confined to field sites in areas already affected by BBW. A modestly equipped screenhouse or glasshouse, with adequate security and precautions to prevent escape of the pathogen, has long been advocated for this work at KARI. Meanwhile, the biotechnology laboratory has been designed to meet national standards for the containment of genetically modified organisms (including bacteria and banana plants) so should be more than adequate for the containment of *Xcm* in detached banana tissues or plantlets if suitable tests can be maintained under laboratory conditions. Work described elsewhere in this report shows that recently weaned tissue-cultured banana plantlets can be used for pathogenicity testing under laboratory conditions, possibly as well as detached banana peduncle sections. Although further tests should be caried out compare results of tests for a range of isolates (and common contaminants) using these materials kept under laboratory conditions with similar tests carried out in banana plants maintained in the field, it should now be possible use pathogenicity on banana plantlets maintained under laboratory conditions as a means for the definitive identification of *Xcm*.

In any pathogenicity testing, particularly when a new technique is being tested and proven, it is important to confirm proof of pathogenicity by satisfying Kochs postulates. This requires demonstration of:

- a consistent association between disease and a specific organism;
- ability of that organism to reproduce symptoms of the disease when inoculated into the plant as a pure culture; and
- consistent reisolation of same organism only from plants showing symptoms of the disease.

Record keeping and reporting

Routine samples received for disease diagnosis or confirmation: KARI is the national laboratory for confirming and recording the diagnosis of BBW so a robust system should be maintained to record all incoming samples and the results of analysis. This should preferably consist of a book (sample day-book) with bound and numbered pages that should not be removed or tampered with. Entries in the book should be made only by designated persons but the book should be available for inspection by anyone who may need to see it, including other field or laboratory workers who may need to check on the identity of cultures or the outcome of diagnostic investigations. Records should include details of each sample (date collected, by whom, any field code or reference, geographic origin or location,) host variety/species, type of tissue collected, disease status and/or brief symptoms, information where (or from whom) more detailed records can be obtained, and a space to record the outcome of laboratory investigations. A short and unambiguous laboratory code should be assigned to each sample. It may be best to have standard record sheet(s) to collect information from the field and laboratory and also for summarising information to be entered into a computerised database, but do not rely on electronic records alone.

Experimental work: The way in which scientists and technicians record experimental work may be subject to NARO policy or guidelines and/or personal preferences. However, it is

good practice to keep records of all activities written down in bound notebooks with pages that are not likely to be torn out or tampered with by others. Loose-leaf folders may be more appropriate for some purposes (such as collating field sample record sheets or records of batches of media prepared). Whatever the method, it is important to record **all** details of experimental planning, materials, methods including modifications to methods or procedures that often arise in the course of experimental work, conditions under which experiments are conducted (temperatures etc.), observations (planned and actual), results, analyses, conclusions and proposals for further work. Of course, records can be greatly simplified for examination of samples by routine or well-established techniques. It is important to keep records available and accessible to those who need to use them – for instance, if more than one person is involved in examining or identifying BBW samples that come into the laboratory then sample and analysis book(s) should be available to, and understood and maintained by, those persons who are designated to carry out the work.

Useful references:

Fahy, P.C. & Gabrielle J. Persley, G.J. (Editors) (1983). Plant Bacterial Diseases: A Diagnostic Guide. Academic Press Inc. (London) Ltd.

Lelliott, R.A. & Stead, D.E. (1987). Methods for the Diagnosis of Bacterial Diseases of Plants. Blackwell Scientific Publications.

Schaad, N.W., Jones, J.B. & Chun, W. (Editors) (2000). Laboratory Guide for Identification of Plant Pathogenic Bacteria. Third edition. American Phytopathological Society.

Waller, J.M., Lenné, J.M. & Waller, S.J. (2002). Plant Pathologist's Pocketbook. Third edition. CABI Publishing.