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DEVELOPMENT OF A SEMI-SELECTIVE MEDIUM FOR *Xanthomonas campestris* pv. *musacearum*

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ABSTRACT

Banana bacterial wilt disease caused by *Xanthomonas campestris* pv. *musacearum* (Xcm) is a serious devastating disease of bananas in Uganda. The disease causes wilting, premature ripening of banana fruits and death of banana plants. There is limited information on the ecology and biology of the pathogen to assist in development of integrated control measures. Studies on the epidemiology of the disease have been limited by lack of a reliable detection method for the pathogen. This paper reports on the progress towards the development of a selective media for the pathogen. Using a pathogen isolate (KY44) and 8 other bacterial strains associated with the pathogen in banana tissues, soil and insect surfaces, a new medium has been developed. The semi-selective medium designated CCA contains (g L^{-1}): yeast extract, 1g; glucose, 1g; peptone, 1g; NH_4Cl, 1g; $MgSO_4$. $7H_2O$, 1g; K_2HPO_4, 3g; beef extract, 1g; cellobiose, 10g; peptone, 1g; agar, 14g; cephalexin, 40mg; 5-fluorouracil, 10mg and cycloheximeide, 120 mg. A combination of cephalexin and 5-Fluorouracil in the media controlled most of the common bacterial strains isolated from soil and plant tissues. Results from preliminary testing for selectivity and recovery from soil and plant tissues of CCA medium using YPGA as the standard indicate that the medium is able to recover 59% of the target bacteria and is selective against 82% of the common bacterial contaminants. CCA medium is therefore a valuable tool that can be useful in epidemiological studies of Xcm pathogen.

Key Words: *Musa* sp., plant tissues, Uganda

RÉSUMÉ

La maladie banana bacterial wilt causée par *Xanthomonas campestris* (Xcm) est une maladie dévastatrice sérieuse de la banane en Ouganda. La maladie produit de la dégénérescence, un mûrissement précoce de fruits de banane et la mort des plantes de banane. Il existe peu d'informations relatives à l'écologie et à la biologie du pathogène en vue d'assister dans le développement de mesures intégrées de contrôle. Les études sur l'épidémiologie de la maladie ont été limitées par le manque d'une méthode fiable de détection du pathogène. Ce papier rapporte sur le progrès vers le développement d'un médium sélectif pour le pathogène. Utilisant un isolé du pathogène (KY44), 8 autres types de bactéries associées au pathogène dans les tissus de la banane, à la surface du sol et des insectes, un nouveau médium était développé. Le médium semi sélectionné, désigné CCA contient (g L^{-1}): extrait de levure, 1g; peptone, 1g; NH_4Cl, 1g; $MgSO_4$, $7H_2O$, 1g; K_2HPO_4, 3g; extrait de bœuf, 1g; cellobiose, 10g; peptone, 1g; agar, 14g; cephalexine, 40 mg; 5-fruorouracil, 10 mg et cycloheximeide, 120 mg. Une combinaison de cephalexine et 5-Fluorouracil dans le médium contrôlait la plupart des types de bactéries isolées à partir de du sol et des tissus de plantes. Les résultats des tests préliminaires pour la sélectivité et la récupération à partir du sol et de tissus de plante de médium CCA en utilisant YPGA comme standard, indiquent que le médium est capable de récupérer 59% de la bactérie-cible et est sélective contre 82% de contaminateurs de la bactérie commune. Le médium CCA est alors un outil important qui peut être utile dans les études épidémiologiques du pathogène Xcm.

Mots Clés: *Musa* sp., tissus de la plante, Uganda

INTRODUCTION

Xanthomonas campestris pv. *musacearum* (herein referred to as Xcm) is the causal agent of banana bacterial wilt disease. This disease was first reported in Ethiopia on enset, a family relative of bananas in 1968 (Yirgou and Bradbury, 1974) and was later found to attack bananas of variety Du casse hybrid (Yirgou and Bradbury, 1974). Out break of this disease in Uganda was first reported in 2001 in Mukono district, central Uganda (Tushemereirwe *et al.*, 2003). They reported that the disease was spreading rapidly with plantation incidence of up to 70% in some places. Bananas are the most important food crop in Uganda according to annual production, consumption rate and agricultural land allocated to the crop (Tushemereirwe *et al.*, 2004). The disease affected both highland and exotic bananas causing losses of up to 70% in a period of 1 year. Symptoms of the disease include wilting and premature ripening of the fruit (INIBAP, 2005). According to Yirgou and Bradbury (1974), the pathogen enters the host through wounds on roots, pseudostems and leaves. It is also suspected that bacteria enter the plant though the inflorescence as reported for bugtok disease (Soguilon *et al.*, 1995). Currently the disease has been confirmed in 34 districts of Uganda distributed in all the four regions in the country.

To control the disease, cultural control practices that include *in situ* destruction of healthy and neighbouring plants plus removal of male buds have been recommended. However there is lack of sufficient information on the ecology and biology of the pathogen to back the recommendation. Epidemiological studies using non-selective media have been difficult because of the slow growth of the pathogen. The pathogen gets out competed by growth of saprophytes on the media surface. Use of selective media developed for other *Xanthomonas campestris* pathovars (Chun *et al.*, 1983; Claftlin *et al.*, 1987; Fessehaie *et al.*, 1999; Juhnke *et al.*, 1989; Pruvosti *et al.*, 2005; Sijam *et al.*, 1991 and Schaad and White, 1974) has not been successful for isolating Xcm. The study was therefore undertaken to develop a selective media for isolating Xcm from soil, infected tissues and insect surfaces. The media will assist in studying the epidemiology of the disease.

MATERIALS AND METHODS

Isolation of Xcm and the associated bacteria. The pathogen isolate designated (KY44) was obtained from an infected banana plant and used for this study. Five bacterial contaminants associated with KY44 in plant tissues and the two most common bacterial strains obtained from soil (Table 1), were also used in the study. To isolate the pathogen, 4g of an infected plant tissue was surface sterilised by dipping in 97% ethanol followed by flame sterilisation. The sterilised tissue was then macerated in 10ml of sterile 0.01M $MgSO_4$ buffer. The resulting suspension was then serially diluted and 0.1ml of each dilution plated on YPGA (Yeast, 5g; Peptone, 5g; Glucose, 4g and Agar, 12g per liter) and incubated at 25°C. After 72h incubation, single colonies of Xcm were transferred to a fresh YPGA media to obtain pure cultures. The pathogenicity of KY44 was confirmed by inoculating banana plants with 0.5ml bacterial suspension per plant (approximately

TABLE 1. Bacterial strains used in the study

Strain	Identity	Source	Gram reaction
020	-	Plant tissues	-
018	*Pseudomonas* spp.	Plant tissues	-
021	-	Plant tissues	-
022	*Klebsiella pneumoniae*	Plant tissues	+
Red	*Arcanobacterium heamolyticum*	Plant tissues	+
S1	*Bacillus pumilis*	Soil	+
S2	*Arcanobacterium heamolyticum*	Soil	+
N	-	Nectar	-
KY44	*Xanthomonas campestris pv. musacearum*	Plant tissues	-

10^8cfu/ml) and observing the plants for development of disease symptoms. The pathogen was re-isolated from diseased plants to complete Koch's postulate.

Bacterial strains from soil were obtained from samples taken from an infected field and rotting compost near homesteads. Ten grammes of this soil was suspended in 100ml of sterile water and the resulting suspension serially diluted. From each of the dilutions, 0.1ml was plated on YPGA medium. To obtain bacteria associated with insect surfaces, nectar was obtained from male flowers from banana plants in fields infected with the disease. From each nectar sample, 10µL was plated on YPGA media and incubated for 48h at 25°C. Strains were separated and where possible identified based on their colour, size and growth characteristics. They were all tested for the gram reaction using 3% potassium hydroxide solution.

Sensitivity to antibiotics. To control the growth of the associated bacteria, various antimicrobial compounds were tested for ability to suppress bacterial growth. The compounds tested include cephalexin, tobramycin, phosphomycin, methyl violet 2B, methyl green, 5-fluorouracil, novobiocin, neomycin sulfate, polymixin, bacitracin, rifampicin, cefazolin, potassium tellurite, nitrofurantoin, pyridoxine HCl, trimenthoprin and erythromycin. The compounds were selected based on their antimicrobial activity spectrum and were evaluated in solid and liquid YPG medium. Cycloheximide was included in the media to suppress fungal growth. These compounds were evaluated for ability to suppress other bacteria without adversely affecting growth of Xcm.

Selection of suitable basal media. A new basal medium was sought because preliminary observations indicated that it was difficult to control growth of many saprophytes using antibiotics in YPGA as a basal medium probably due to its nutrient composition. Therefore, a new basal media with different nutrient composition but favouring the growth of Xcm was needed. To obtain a suitable basal medium, 5 basal media that have been developed for other *Xanthomonas campestris* pathovars were evaluated for suitability for growth of Xcm. These include YPGA, SX (Schaad and White, 1974), SM (Chun *et al.*, 1983), CKTM (Sijam *et al.*, 2001), CTA (Fessehaie *et al.*, 1999) and MXP (Claftlin *et al.*, 1987). The above media were plated with 20µl per plate of cell suspension containing about 10^8cfu/ml and incubated at 25°C for up to 120h and monitored. Selection of a suitable media was based on the growth rate as determined from the diameter of the colonies measured after 72h, 96h and 120h incubation, colony colour and distinctiveness of the colonies.

Formulation and testing selectivity of the new medium. Using the most promising media selected above, antibiotics were added and the medium evaluated for its selectivity. To determine medium selectivity, 0.1ml aliquot of the bacterial suspension was transfered into 2g of non-sterile soil obtained from a banana field. The inoculated soil was serially diluted and 20µL of the dilutions plated on triplicate solid agar plates of both YPGA and the new medium. To determine selectivity when isolating from plant tissues, 10g of an infected banana tissue was macerated in 10ml of sterile distilled water. The resulting suspension was serially diluted and 20µl of the dilutions plated on both the new and YPGA media. After 3-7 days of incubation, the proportion of the colonies of the target bacteria to the total number of bacterial colonies per plate for the two media was determined.

Data analysis. Means of colony counts and diamaters were subjected to ANOVA using general linear model procedure of SAS (SAS Institute Inc., 1997). Significant colony counts and diameter means were separated using Waller-Duncan K-ratio t-test at 0.05 level of significance.

RESULTS

The fastest growth rate was recorded on YPGA and the slowest growth rate was recorded on SM and MXP media while CTA, CKTM and SX were intermediates. Colonies of Xcm appeared after 48h incubation on YPGA media but appeared after 76 hours on CKTM, SX, MXP,CTA and SM. Little growth was observed on SM and MXP after 72h incubation (colony diameters were 0.2mm and 0.3mm respectively compared to

>1mm on CTA, CKTM and YPGA. The sizes of the colonies only increased to 1.02 and 1.12mm respectively when the incubation period was increased to 120h compared with the 2.44mm on CKTM, 3.30mm on CTA and 4.62mm colony diameter obtained on YPGA. The mean colony diameter on YPGA at 120h incubation was significantly different from the colony diameter obtained with other media (LSD=0.5). Differences in mean colony diameters among CKTM, CTA and SX were highly significant. Differences in mean colony diameters between MXP and SM were not significant but were significantly different from colony diameters obtained with other media. *X. c.* pv. *musacearum* colonies appeared white even after 120h incubation on MXP and SM. They were yellow on CTA, MXP and CKTM, although the pigmentation was not as intense as on YPGA medium (Fig. 1). Apart from YPGA, colony growth rate was faster on CTA media compared with other media (Table 2). The plating efficiencies for Xcm of the above five basal media were compared with that of YPGA. The results indicate that CKTM has the lowest plating efficiency followed with CTA but MXP media showed the highest level of plating efficiency. In addition CTA medium had higher recovery efficiencies and the colour of Xcm appeared more intense than CKTM medium (Table 2). Selection for the best basal media was mainly based on the time taken for colonies to appear after plating on solid media, the rate of colony growth and plating efficiency. Based on the faster growth rate, the comparatively high plating efficiency, distinctiveness of colonies and the yellow pigmentation typical of Xcm on non-selective YPGA media, CTA was selected as the basal media. In this trial however, trehalose was substituted with cellobiose, and therefore we have designated it CCA. This medium contains ($g L^{-1}$); glucose, 1g; yeast extract, 1g; peptone, 1g; ammonium chloride, 1g; K_2HPO_4, 3g;

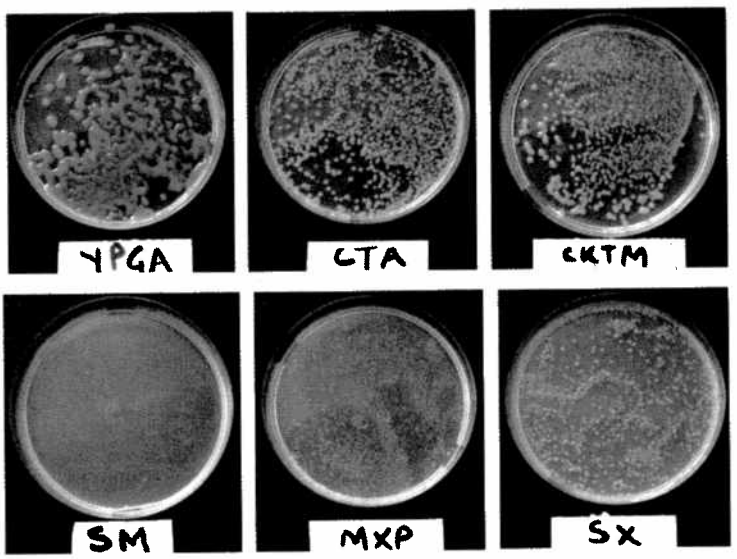

Figure 1. Colony characteristics of Xcm on YPGA (A), CTA (B), CKTM (C), SM (D), MXP (E) and SX (F) agar media.

$MgSO_4.7H_2O$, 1g; cellobiose, 10g and agar, 14g.

Preliminary screening of 18 compounds listed in Table 3 showed that rifampicin (0.3mg L^{-1}), polymixin sulfate(100mg L^{-1}), methyl violet 2B(1ml L^{-1}), novobiocin (15mg L^{-1}), neomycin sulphate (15mg L^{-1}) and bacitracin (0.2mg L^{-1}) strongly suppressed/inhibited growth of Xcm and were therefore not considered for further testing. Methyl green, tobramycin, pyridoxine HCl and nitrofurantoin had no suppressive effect on growth of Xcm and its associated bacterial strains (Table 3) and were also not evaluated further. Three compounds, cephalexin, 5-Fluorouracil and cefazolin showed suppressive effects on the

TABLE 2. Evaluation of *Xanthomonas campestris* pv. *musacearum* colony growth on five solid basal media tested and their plating efficiencies as compared with growth on YPGA

Media	Mean colony diameter at 72h (mm) †	Mean colony diameter at 120h (mm) †	Mean No. of colonies‡	Plating efficiency (%)	Colony colour
YPGA	2.0 ± 0	4.62 ± 0.52	2452 ± 390	100	Yellow
CKTM	1.5 ± .05	2.44 ± 0.26	1006 ±19.8	41.0	Yellow
CTA	1.0 ± 0	3.30 ± 0.66	1438 ± 718	58.6	Yellow
SX	0.5 ± 0	2.02 ± 0.04	1542 ± 25	62.9	Yellow
SM	0.2 ± 0	1.02 ± 0.04	1496 ± 633	61.0	White
MXP	0.3 ±0	1.12 ± 0.11	1748 ± 131	71.0	White

‡Values indicate the mean number of colonies based on three plates
†Values indicate the mean colony diameters of five isolated randomly selected colonies obtained from two plates

TABLE 3. Susceptibility of selected bacterial strains to selected antibiotics and growth inhibitors

Antibiotic	Bacterial strains								
	018	020	021	022	Red	S1	S2	N	Xcm
Cephalexin (40mg/L)	-	-			-			**	-
Cephalexin (50 - 65mg/L)	-	-		*	*			**	*
Methyl green(2ml/L)	-	-	-	-	-	-	-		-
Methylviolet 2B(1ml/L)	-	-	-	-	-	-			*
PolymixinB sulfate(100mg/L)	-	-			-				**
Bacitracin (0.2mg/L)	-	-			-	**			**
Neomycin sulfate(15mg/L)	*	*				*			**
Rifampicin (0.3mg/L)	*	*			**		**	**	**
Rifampicin (10 -15mg/L)	**	**			**				**
Phosphomycin (5mg/L)	-	-	-	-	-	*	-	*	**
5-Fluorouracil (10mg/L)	*	*		*					-
5-Fluorouracil (18 - 40mg/L)	*	*			*				*
Erythromycin (10mg/L)	-	-			-	-	-	*	*
Trimenthoprin (30mg/L)	-	-			-	-			**
Novobiocin (15 - 25mg/L)						**		**	**
Tobramycin (1.8mg/L)	-	-			-			*	-
Tobramycin (3.0- 4.5mg/L)	-	-			-			**	-
Pyridoxine HCl (1mg/L)	-	-				-		*	-
Amoxicillin (10mg/L)	-	-				*		**	*
Nitrofurantoin (10mg/L)	-	-	-		-	-		**	-
Potassium tellurite (15mg/L)	-				-	-			
Cefazolin (25mg/L)			-	*			*		-

- Growth of the bacteria strain not affected
* Growth of the bacteria strain slightly suppressed
** Growth of the bacteria strain highly suppressed

TABLE 4. Comparative selectivity of CCA media for isolation from soil inoculated with Xcm cells

CFU on CCA[x]		CFU on YPGA[x]		% of Xcm on CCA	% of Xcm on YPGA
Xcm	Total bacteria	Xcm	Total bacteria		
2778±996	3397 ±1404	2772±1180	6580± 2150	82	42

x- average number of colonies per plate from three plates

associated strains and were selected for further evaluation to obtain good working concentrations (Table 3). 5-Fluorouracil inhibited Xcm growth at 18 - 40mg L^{-1} concentrations but at 10mg L^{-1} growth of the fast growing strains (018, 020 and 022) was significantly suppressed while growth of Xcm was not affected. Concentrations above 50mg L^{-1} of cephalexin suppressed Xcm but at 40mg L^{-1}, Xcm growth was not suppressed while that of bacterial strains N and Red was controlled. Growth of strains 022 and S_1 was suppressed when 25 mg L^{-1} of Cefazolin was added to the medium. These were therefore included in the formulation of the new media. When 5-Fluorouracil (10mg L^{-1}), cephalexin (40mg L^{-1}) and Cefazolin (25 mg L^{-1}) were incorporated in the new media, in different combinations and tested for capacity to inhibit the bacterial strains in Table 1, a combination of cephalexin and 5-Fluorouracil was highly suppressive while allowing Xcm to grow. The results were not significantly different when cephalexin was substituted with cefazolin in the media. Cycloheximide was added to inhibit fungal growth. The media appeared to be highly toxic when all the three compounds were added to the media at the same time and in addition, highly suppressed growth of Xcm colonies. Colonies of Xcm appeared on this medium as yellow, round and convex and appeared after 5-7 days of incubation. All antibiotics were added as filter sterilised stock solutions. When the selectivity of CCA was evaluated by comparing with YPGA media as the standard, CCA was 82% selective for Xcm compared with 42% for YPGA.

DISCUSSION

This study was initiated because of lack of selectivity of YPGA medium that was used for isolation of the pathogen and also to assist in monitoring the spread of the pathogen in the environment. It was difficult to isolate the pathogen from artificially infested soil and infected tissues that are in advanced stages (wilted) of infection due to high levels of contamination. The new media has been designated CCA and includes two antimicrobial compounds cephalexin and 5-Fluorouracil and one antifungal compound cycloheximide. 5-Fluorouracil and cephalexin at 10mg L^{-1} and 40mg L^{-1} respectively, suppressed growth of most of the antagonistic bacteria from soil and plant tissues and allowed colonies of Xcm to grow. The two compounds suppressed both gram positive and gram negative bacterial strains. Although a combination of cephalexin and 5-Fluorouracil gave better results, substitution of cephalexin with cefazolin did not significantly alter the selectivity of the medium and growth of Xcm colonies. Cefazolin can therefore be a good substitute for cephalexin in the media. Cycloheximide inhibited most fungal growth. Preliminary tests indicate that CCA is 82% selective and it can recover up to 59% of the target bacteria. Most bacterial contaminants seem to readily utilise glucose and peptone as substrates for carbon and nitrogen and were therefore avoided while developing a selective medium. CCA medium uses cellobiose as a carbon source with very little amount of glucose (1g L^{-1}). Growth of Xcm colonies on MXP, SX and SM was very slow because Xcm does not hydrolyse starch and triphenyl-tetrazolium chloride (Bradbury, 1986). *X. c.* pv. *musacearum* colonies on this medium (CCA) are yellow, convex and mucoid with an even colony margin.

Cultural characteristics of Xcm colonies on YPGA and on the new CCA medium make them easily distinguishable from other white pigmented bacteria. The yellow colour is not as intense as in YPGA medium but the intensity increases with the size and age of the colonies. The amount of

time needed for colonies to appear on CCA medium is 4-5 days compared with 3 days for YPGA. Although the growth rate is reduced, the media is better than YPGA because it allows selective isolation and quantification of Xcm from soil and rotting tissues which is difficult with YPGA medium. The media allowed growth of Xcm isolated from cabbage and broccoli but the colonies remained so small (0.2 - 1mm diameter) even after 72h incubation. CCA is therefore a valuable tool in epidemiological studies of Xcm.

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