European Journal of Plant Pathology **110:** 991–1001, 2004.

A diagnostic medium for the semi-selective isolation and enumeration of *Xanthomonas axonopodis* pv. *vignicola*

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Accepted 10 May 2004

Key words: cefazoline cellobiose methionine medium, *Vigna unguiculata*, *Xanthomonas campestris* pv. *vignicola*

Abstract

A semi-selective medium for isolation of *Xanthomonas axonopodis* pv. *vignicola* from cowpea (*Vigna unguiculata*) plant and soil samples was developed. Twelve carbon and five nitrogen sources were tested with four strains of *X. axonopodis* pv. *vignicola*, and 25 antibiotics were screened against saprophytes. D-cellobiose (10 g) was selected as the optimal carbon source. Among the antibiotics, cefazoline inhibited growth of most of the saprophytes with little effect on strains of the pathogen. D,L-methionine enhanced growth of *X. axonopodis* pv. *vignicola*. Boric acid along with ammonium chloride suppressed growth of *Pseudomonas fluorescens*. The semi-selective medium designated as *cefazoline-cellobiose-methionine* (CCM) medium contained K_2HPO_4 1.34 g, KH_2PO_4 0.4 g, $MgSO_4$ 0.3 g, H_3BO_3 0.2 g, NH_4Cl 1.0 g, D-cellobiose 10 g, cycloheximide 0.2 g, D,L-methionine 1.0 g, cefazoline 10 mg and agar 14 g per l of water (pH 7.2). Colonies of *X. axonopodis* pv. *vignicola* on CCM medium were whitish, round, raised and 0.2–1.8 mm in diameter 96 h after incubation. CCM medium generally inhibited growth of *Pantoea agglomerans*, *Bacillus subtilis* and saprophytes isolated from cowpea leaves. Colonies of *Pseudomonas fluorescens* and a saprophytic bacterium, which were not completely suppressed by CCM, could be differentiated from *X. axonopodis* pv. *vignicola* by their smaller size and different color. The CCM medium proved useful for isolation of *X. axonopodis* pv. *vignicola* from cowpea plant and soil samples. This is the first report of a semi-selective medium developed for detection of *X. axonopodis* pv. *vignicola.*

Introduction

Bacterial blight of cowpea (*Vigna unguiculata*) caused by *Xanthomonas axonopodis* pv. *vignicola* (Vauterin et al., 1995), formerly *X. campestris* pv. *vignicola* has been reported in North America (Burkholder, 1944), Latin America (Vakili et al., 1975), Asia (Raj and Patel, 1977), Africa (Kaiser and Ramos, 1979) and Europe (Severin and Stancescu, 1990). The disease begins with tiny, water-soaked spots on leaves that gradually develop into necrotic lesions. Then, the pathogen systemically invades leaves, stems and seeds of cowpea plants (Sikirou, 1999a). Stems and seeds from symptomless plants may harbour *X. axonopodis* pv. *vignicola* externally as well as internally (Patel and Singh, 1984). Bacterial blight infected seeds reduce stand emergence (15–67%) and

increase plant mortality (up to 81%). Yield losses up to 92% occur, depending upon cultivar and plant growth stage when infected (Kishun, 1989). In severe cases, entire fields are completely destroyed (Sherwin and Lefebvre, 1951). Seeds are an important inoculum source while contaminated plant debris and insect vectors seem to be less important (Sikirou, 1999b). Control methods consist of resistant varieties, intercropping, shift of sowing date and sanitary measures (Sikirou et al., 2001; Wydra et al., 2001).

To develop control methods and monitor their effect, as well as to test seed lots for contamination, a reliable and sensitive detection method is needed. Isolation and identification of bacteria from plants and soil is difficult due to high numbers of saprophytic bacteria (Khatri-Chhetri, 1999). Saprophytes may be similar in colony color and shape and may also inhibit growth of *X. axonopodis* pv. *vignicola*. Semi-selective media are useful for isolation of bacterial pathogens in plant tissue and soil (Wang et al., 1999; Toussaint et al., 2001). Wang et al. (1999) reported on a semi-selective medium (m-XAM) for isolation of *X. albilineans* as being more sensitive, especially from symptomless tissue, than immunological methods and PCR.

Complete media such as YDC (Schaad and Stall, 1988) are used for detection of *X. axonopodis* pv. *vignicola*. No specific medium for the isolation of *X. axonopodis* pv. *vignicola* has been reported. The objective of this study was to develop a semi-selective medium for isolation and quantification of *X. axonopodis* pv. *vignicola* from cowpea plant and soil samples.

Materials and methods

Bacterial strains

Xanthomonas axonopodis pv. *vignicola* strains were isolated from diseased cowpea leaves collected in Benin, Nigeria, Niger and Mozambique (Table 1) and characterized for their pathogenic, biochemical and genetic characteristics (Khatri-Chhetri et al., 1998; Verdier et al., 1998; Khatri-Chhetri, 1999). Additionally, a reference culture from Brazil was obtained. Four saprophytes (SP), SP1 (white, flat, large, wrinkled colonies, Gram-positive), SP2 (red, small colonies, Gram-positive), SP3 (dark yellow, large, colonies, Gram-negative) and SP4 (creamy white, large, very slimy colonies, Gram-negative), were isolated from cowpea leaves from Benin and Nigeria along with *X. axonopodis* pv. *vignicola* strains. *Pseudomonas fluorescens* (GSPB 1713), *Pantoea agglomerans* (former *Erwinia herbicola*) (GSPB 2314) and *Bacillus subtilis* (GSPB 25) were obtained from Göttinger Sammlung Phytopathogener Bakterien (GSPB), Göttingen, Germany. Strains were maintained on yeast glucose chalk agar (yeast extract 10.0 g, D-glucose 20.0 g, precipitated chalk 20.0 g, Oxoid agar No.3 12.0 g, demineralized water 1 l, pH adjusted to 7.2) at 14 °C (Stead, 1990). For long-term preservation, strains were lyophilized and deposited in the GSPB collection.

Selection of ingredients

Eleven carbon sources selected from 95 organic substrates were tested for their metabolization by *X. axonopodis* pv. *vignicola* strains using the Biolog

Table 1. Origins and sources of strains of *X. axonopodis* pv. *vignicola*

Designation of strains	Origin	Source[a]
4, 12a2, 16g (2517)[b]	Benin	Sikirou (1997)
19bg (2407), 23a, 28a1g (2408), 30a1 (2409), 35a3 (2411), 39a4	Nigeria	Wydra (1997)
12a2, 43b2, 44b1, 49a (2413), 51b3 (2415), 56a (2590), 59a (2416)	Niger	Wydra (1997)
1b2 (2402)	Cameroon	Wydra (1997)
FNPB1 (2417)	Mozambique	Rao (1997)
536a1g	Venezuela	Rudolph (1987)
NCPPB 3187 (2332)	Brazil	NN
267-5 (2451), 267-10 (2454), 268-10 (2458), 317-2 (2459)	Thailand	Furuya (1996)

[a]FNPB1 obtained from Y.P. Rao, Maputo, Mozambique; NCPPB = National Collection of Plant Pathogenic Bacteria, Hatching Green, Harpenden, UK; NN = unknown; reference strains originating from Thailand were obtained from N. Furuya, Japan Culture Collection (MAFF), Kyushu University, Japan.
[b]GSPB (Göttinger Sammlung Phytopathogener Bakterien) number.

System (GN MicroPlate, Biolog Inc., Hayward, USA) (Khatri-Chhetri et al., 2003). Eight of these carbon sources (D-cellobiose, dextrin, D-fructose, D-maltose, methyl pyruvate, methyl succinic acid, sucrose and D-trehalose) were metabolized by all strains, glycerol by 93–96% of the strains, lactulose by only two strains that were isolated from pustule symptoms in Mozambique, and α-D-lactose was not utilized by any strains. Starch, not included in the previous Biolog studies was also tested. Four amino acids (D-alanine, L-glutamic acid, L-serine and L-threonine) used by most strains in the Biolog test and D, L-methionine were tested for their ability to enhance growth of *X. axonopodis* pv. *vignicola*. Ammonium chloride, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, peptone and potassium nitrate were used as nitrogen sources. Boric acid was tested to suppress growth of saprophytes and cycloheximide for the inhibition of fungal growth.

Preparation of media

The following ingredients were used as a basic medium: K_2HPO_4 (1.34 g l^{-1}), KH_2PO_4 (0.4 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.3 g l^{-1}), cycloheximide (0.2 g l^{-1}) and agar (14.0 g l^{-1}). To test components, nitrogen sources (1 g l^{-1}), boric acid (0.2 g or 0.3 g l^{-1}) and ingredients of the basic medium (except cycloheximide) were suspended in demineralized water, and the pH adjusted to 7.2 by HCl/NaOH. All carbon sources, amino acids, cycloheximide and cefazoline were dissolved in distilled water, partly prewarmed below 50 °C for dissolving, filter-sterilized and added to the medium after autoclaving and cooling to ca. 50 °C. Twelve carbon sources, five amino acids (10 and 1 g l^{-1}, respectively) and three concentrations of sucrose (8, 10 g and 12 g l^{-1}) were used. Nutrient glucose agar (NGA) consisting of nutrient broth (8 g), glucose monohydrate (11 g), yeast extract (3 g) and agar (14 g per l of demineralized water) was used as the complete medium for comparison purposes.

Preparation of bacterial cultures

Colonies of each *X. axonopodis* pv. *vignicola* strain and saprophyte, grown for about 48 h on NGA, were suspended in sterile 0.01 M $MgSO_4$. A bacterial concentration of about 10^8 colony forming units (CFU)/ml (i.e. an optical density of 0.06 at 660 nm) was used. One hundred microliters of suspensions of serial, 10-fold dilutions were plated per Petri dish in two replicates. The Petri dishes were incubated at 28–29 °C and observed daily up to 96 h after incubation. Bacterial growth was usually counted at the 10^{-5} dilution which corresponded to approximately 100 CFU/Petri dish.

Evaluation of ingredients in combination

Twelve carbon sources (cellobiose, fructose, sucrose, dextrin, glycerol, lactose, lactulose, maltose, methylpyruvate, methylsuccinate, starch, trehalose, all supplied by Sigma, Germany) with NH_4Cl as nitrogen source, and five nitrogen sources (NH_4Cl, $(NH_4)SO_4$, $(NH_4)_2PO_4$, KNO_3, peptone) with glucose (10 g l^{-1}) as carbon source were evaluated against *X. axonopodis* pv. *vignicola* strains (16g, 28a1g, 49a, FNPB1) and saprophytes (*B. subtilis*, *P. agglomerans*, *Ps. fluorescens*, SP1, SP2, SP3, SP4) to select the optimum carbon and nitrogen sources, respectively. After preliminary screening, D-cellobiose and sucrose (10 g l^{-1}) as carbon sources and NH_4Cl, $(NH_4)SO_4$, and $(NH_4)_2PO_4$ (1 g/l) as nitrogen sources were further tested. Five amino acids (methionine, alanine, threonine, glutamic acid, serine, all supplied by Sigma, Germany) (1 g l^{-1}) were tested with the combination of D-cellobiose (10 g l^{-1}) and NH_4Cl (1 g l^{-1}). Growth was evaluated by measuring colony diameter.

Test of antibiotics

Antibiotic impregnated paper discs (6 mm) (Becton Dickinson and Hoechst, Germany) of 23 antibiotics (numbers in brackets indicate μg antibiotic per disc): ampicillin (AM 10), amoxicillin (AMC 30), cefazoline (CZ 30), cefixim (CFM 10), cefotaxim (CTX 30), cefpirom (CPO 30), cefuroxim (CXM 30), ciprofloxacin (CIP 5), erythromycin (E 15), fusidinic acid (FA 10), imipinem (IPM 10), lincomycin (L 15), meropenem (MEM 10), metronidazole (MTZ 5), mezlocillin (MZ 30), neomycin (N 30), penicillin G (P 10), piperacillin (PIP 30), rifampicin (RD 2), tazobac (TZ, mixture of piperacillin PIP 30 + tazobactam T 10), teicoplanin (TEC 30), tetracyclin (TE 30) and vancomycin (VA 30) were evaluated. The paper discs were applied to the agar surface of NGA medium after plating the pathogen suspension, and the Petri dishes were kept under cool conditions (7 °C) to allow antibiotics to

diffuse into the agar prior to bacterial growth. The radius of the inhibition zone was measured 48 h after incubation and means were calculated from two replications. Additionally, powder formulations of fosfomycin (FM, 2.5 mg l^{-1}) and tobramycin (TM, 0.4 mg l^{-1}) were dissolved in 50% ethanol, filter-sterilized and added to the cooled medium. The medium was poured into Petri dishes, and after drying for 24 h inoculated with bacteria (100 µl at 10^{-4} dilution or 2×10^4 CFU ml^{-1} per Petri dish) with two replications. Bacterial growth was recorded 48 h after incubation. Reactions were grouped according to the radius of inhibition zone 48 h after incubation, if not otherwise stated: S (susceptible) 5–20 mm, MS (medium susceptible) 3–5 mm, R (resistant) 0–2 mm.

Evaluation of medium CCM

Growth of 21 strains of *X. axonopodis* pv. *vignicola* was evaluated on CCM and NGA. Bacterial suspensions of OD 0.06 (660 nm) were serially 10-fold diluted and 100 µl were plated from three dilution levels (10^{-4}, 10^{-5}, 10^{-6}). The trial was performed with two replicates. Efficiency of the semi-selective medium was quantified according to the following formula: Efficiency $\% = [(\text{CFU/CCM medium})/(\text{CFU/ NGA})] \times 100$.

The CCM medium was tested by adding a suspension of *X. axonopodis* pv. *vignicola* mixed with three (SP2, SP3 and *P. fluorescens*) or four (SP1, SP4, *B. subtilis* and *P. agglomerans*) saprophytes. Pathogen and saprophyte suspensions of OD 0.06 corresponding to approximately 10^8 CFUml^{-1} were mixed equally and then 10-fold serially diluted to 10^{-5}. Cowpea exhibiting bacterial blight symptoms from cowpea fields in Benin and Nigeria, naturally infected seed samples (genotypes IT92KD-257-7, IAR48) from Kano, Nigeria and soil samples from cowpea fields in Benin (Cotonou and Ina, southern and northern Benin, respectively) and Nigeria (Ibadan, southwestern Nigeria) were evaluated for recovery of *X. axonopodis* pv. *vignicola* on CCM and NGA. Due to fast and slimy growth of bacteria and coalescence of colonies on NGA, and slow growth on CCM, observations were made 48 and 96 h after inoculation, respectively. Bacterial numbers were counted when possible, or scored as +++ (uncountable number of colonies), ++ (many colonies) and + (few colonies, flowing together).

For isolation from leaf samples, small pieces from the border of lesions were macerated in 0.01 M $MgSO_4$ and 10-fold serial dilutions prepared. For recovery from cowpea seeds, seeds were soaked overnight at 18 °C in sterile 0.01 M $MgSO_4$ solution. The supernatant was filtered through cheesecloth and then centrifuged at $5200 \times g$ at 15 °C for 20 min. The pellet was resuspended in 5 ml sterile 0.01 M $MgSO_4$ and serially 10-fold diluted.

To recover *X. axonopodis* pv. *vignicola* from soil samples, the quantity of soil used in the tests was optimized. Bacteria were isolated from 1, 2 and 4 g soil originating from Ina (southern Benin). The three samples were suspended in 5 ml 0.01 M $MgSO_4$ solution and serially 10-fold diluted to 10^{-6}. A suspension (100 µl) was plated from each dilution level on CCM medium and NGA with two replications. Satisfactory results were achieved with 1 g soil, and this quantity was used in subsequent tests. To investigate the effect of soil on growth of the pathogen, 1 g of native soil that originated from Cotonou and Ina (Benin) was mixed with 0.5 ml suspension (2.3×10^8 CFU ml^{-1}) of *X. axonopodis* pv. *vignicola* (strain 16 g). The samples were suspended in 4.5 ml 0.01 M $MgSO_4$ and 10-fold diluted up to 10^{-6}. Dilution levels of 10^{-3}–10^{-6} (100 µl) were plated on NGA and CCM with two replications. Strain 16 g at dilution levels of 10^{-5} and 10^{-6} were plated (100 µl) on NGA and CCM as a control. Petri dishes were incubated at 28–29 °C and observed daily until 96 h after incubation. Growth was evaluated as described above.

Statistical analysis

Means and standard errors were calculated; bacterial numbers were log-transformed and analyzed by ANOVA with *F*-test using SAS/STAT Software (SAS Institute Inc., 1997).

Results

Selection of carbon and nitrogen sources

D-Cellobiose promoted growth of the four *X. axonopodis* pv. *vignicola* test strains and suppressed growth of *P. fluorescens* and *B. subtilis*, while among the other 11 C-sources, the pathogen generally grew well on fructose, sucrose and lac-

tose. Among the latter C-sources only lactose suppressed growth of *P. fluorescens*, while *B. subtilis* was slightly suppressed by nearly all C-sources except lactulose and trehalose. *X. axonopodis* pv. *vignicola* did not grow on starch, while no growth of any strain was observed on methylpyruvate and methylsuccinate.

Four nitrogen sources, NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and peptone supplemented to the basal medium supported growth of *X. axonopodis* pv. *vignicola* (strains 16 g, 28a1g, 49a, FNPB1), while KNO_3 inhibited growth completely (data not shown). N-sources had no selectivity against the saprophytes *B. subtilis*, *P. agglomerans*, *P. fluorescens*. In evaluations of cellobiose or sucrose in combination with NH_4Cl or $(NH_4)_2HPO_4$, no clear differences in growth promotion of the pathogen and suppression of the saprophytes (*P. agglomerans*, *Ps. fluorescens*, SP1, SP2, SP3, SP4) were observed (data not shown). Ammonium chloride was selected as N-source.

Evaluation of boric acid and amino acids

Two levels of boric acid (0.2, 0.3 g l^{-1}) were evaluated in basal medium containing NH_4Cl as N-source and sucrose as C-source in comparison to NGA and to basal medium without boric acid, with *X. axonopodis* pv. *vignicola* and saprophytes (*B. subtilis*, *P. agglomerans*, *Ps. fluorescens*) (data not shown). Both concentrations of boric acid reduced growth of *Ps. fluorescens* by 50%, but did not influence growth of the other saprohphytes (*B. subtilis*, *P. agglomerans*) nor of *X. axonopodis* pv. *vignicola* (strains 16g, 28a1g, 49a, FNPB1). Three levels of sucrose (8, 10, 12 g l^{-1}) tested in combination with boric acid did not further promote growth of the pathogen. Boric acid at 0.2 g l^{-1} was chosen as ingredient for the new medium. Any one of five amino acids (D,L-methionine, alanine, threonine, glutamic acid and serine) in a medium with optimal concentrations of D-cellobiose, NH_4Cl and boric acid, D,L-methionine (1.0 g l^{-1}) slightly promoted colony number and size of *X. axonopodis* pv. *vignicola* strains (data not shown).

Selection of antibiotics

Among 25 antibiotics, cefazoline completely inhibited growth of the saprophytes *B. subtilis* and *P. agglomerans*, with no or little (inhibition zone 2 mm, R) effect on *X. axonopodis* pv. *vignicola* strains 16g and FNPB1, and 28a1g and 49a, respectively (Table 2). Saprophytes from cowpea leaves collected in Benin, were totally (SP1, inhibition zone 19 mm, S; SP4, 12 mm, S), slightly (SP3, 1 mm, R), or not (SP2, R) inhibited. Other antibiotics inhibited either growth of all or none of the bacteria tested, or of only the pathogen. By supplementing five concentrations of cefazoline (5, 10, 15, 20 and 25 mg l^{-1}) to a medium containing the selected and optimized quantities of the other constituents, number and size of *X. axonopodis* pv. *vignicola* colonies were drastically reduced at >15 mg cefazoline l^{-1}, whereas the influence on growth of SP2 and *P. fluorescens* was weak at 5 mg l^{-1} (data not shown). Therefore, cefazoline at 10 mg l^{-1} was selected as the optimal concentration.

The semi-selective medium designated as *cefazoline cellobiose methionine* (CCM) medium contained the following components per liter: K_2HPO_4 1.34 g, KH_2PO_4 0.4 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, boric acid 0.2 g, NH_4Cl 1.0 g, agar 14.0 g, D-cellobiose 10.0 g, D,L-methionine 1.0 g, cycloheximide 0.2 g and cefazoline 10 mg, pH adjusted at 7.2. The latter four ingredients were filter-sterilized and pre-warmed before supplementing to the autoclaved medium.

Evaluation of CCM medium

Growth of pure cultures of X. axonopodis *pv.* vignicola

Twenty-two *X. axonopodis* pv. *vignicola* strains from different geographic origins grew on CCM with a mean plating efficiency of 109.3%, ranging from 45.6% (strain 44b1) to 188.6% (strain 28a1g) compared to the colony number on NGA (100%) (Table 3). Colonies of *X. axonopodis* pv. *vignicola* on CCM medium were whitish, round and raised. Colony size was smaller on CCM medium, ranging from 0.2 to 1.8 mm in diameter (mean colony size 0.7 mm) at 96 h after incubation, while colony size on NGA was 0.5–3 mm (mean colony size 1.8 mm) 48 h after incubation.

Growth of X. axonopodis *pv.* vignicola *mixed with saprophytes*

When *X. axonopodis* pv. *vignicola* was grown together with three or four saprophytes, the number of saprophyte CFUs were 7–100% lower on CCM

Table 2. Inhibition of growth of four *X. axonopodis* pv. *vignicola* strains and three saprophytes by 25 antibiotics on NGA medium

Antibiotics	Reaction of bacterial strains						
	Xav[a] 16g	*Xav* 28a1g	*Xav* 49a	*Xav* FNPB1	*B. subtilis*[b]	*P. agglomerans*	*Ps. fluorescens*
Ampicillin 10[c]	R[d]	S	S	R	S	S	R
Amoxicillin 30	MS	S	S	S	S	S	R
Cefixim 10	R	S	S	S	S	S	S
Ciprofloxacin 5	S	S	S	S	S	S	S
Cefpirom 30	R	S	S	MS	S	S	S
Cefotaxim 30	MS	S	S	S	S	S	S
Cefuroxim 30	R	S	S	MS	S	S	R
Cefazoline 30[e]	R	R[f]	R[f]	R	S	S	R
Erythromycin 15	S	S	S	S	S	R	R
Fusidinic acid 10	MS	MS	S	S	S	R	S
Fosfomycin[g,h]	S	R	R	R	MS	MS	MS
Imipinem 10	S	S	S	S	S	S	S
Lincomycin15	R	R	R	R	R	R	R
Meropenem 10[i]	MS	MS	S	R	MS	MS	R
Metronidazole 5	R	R	R	R	R	R	R
Mezlocilllin 30	R	S	S	S	S	S	R
Neomycin 30	R	MS	MS	S	MS	MS	R
Penicillin G 10	R	S	S	R	S	R	R
Piperacillin 30	R	S	S	S	S	S	MS
Rifampicin 2	S	S	S	S	S	MS	MS
Tetracyclin 30	S	S	S	S	S	S	S
Teicoplanin 30	MS	S	MS	MS	S	R	R
Tobramycin[g,h]	R	R	R	MS	S	MS	MS
Tazobac	MS	S	S	S	S	S	MS
Vancomycin 30	S	S	S	S	S	R	R

[a]*Xav* = *X. axonopodis* pv. *vignicola*.
[b]B = *Bacillus*, P = *Pantoea*, Ps = *Pseudomonas*.
[c]Antibiotics supplied on paper disc; numbers indicate μg antibiotic per disc.
[d]Radius of inhibition zone 48 h after incubation: S (susceptible) 5–20 mm, MS (medium susceptible) 3–5 mm, R (resistant) 0–2 mm.
[e]On cefazoline inhibition zones of saprophytes SP1, SP2, SP3 and SP4 were 19 mm (S), 0 mm (R), 1 mm (R), and 12 mm (S), respectively.
[f]Growth inhibition zone 2 mm.
[g]Powder was dissolved in 50% ethanol and sterile-filtered into the medium in a separate experiment.
[h]On fosfomycin and tobramycin saprophytes SP1, SP2, SP3 and SP4 were not inhibited (R).
[i]On meropenem inhibition zones of saprophytes SP1, SP2, SP3 and SP4 were 0 mm (R), 0 mm (R), 8 mm (S) and 0 mm (R), respectively.

than on NGA (Table 4). The colony size of the saprophytes (SP3, *P. fluorescens*) was reduced from 3.0 and 5.0 mm to 0.2 and 1.0 mm, respectively. When four other saprophytes (SP1, SP4, *B. subtilis*, *P. agglomerans*) were added, they were completely inhibited on CCM, and *X. axonopodis* pv. *vignicola* was easy to detect (Figure 1A). In the presence of the saprophytes, *X. axonopodis* pv. *vignicola* failed to grow on NGA. On NGA the colony size of *P. fluorescens* (5 mm) exceeded the colony size of *X. axonopodis* pv. *vignicola* (3–5 mm), while on CCM *P. fluorescens* and SP3 had a lower colony number with a reduced colony size (1 and 0.2 mm, respectively) compared to *X. axonopodis* pv. *vign*icola (1.0–1.5 mm). Saprophyte SP2 did not grow on CCM. Additionally, the color of *P. fluorescens* and SP3 colonies were purplish and yellowish, respectively, while *X. axonopodis* pv. *vignicola* was whitish and could be easily distinguished on CCM medium 96 h after incubation.

Isolation of X. axonopodis *pv.* vignicola *from cowpea plants and seeds*

The pathogen was isolated on CCM from cowpea leaf and stem samples collected in Benin and from seeds originating from Nigeria (Table 5). The pathogen was easily identifiable from all the

Table 3. Plating efficiency of 22 *X. axonopodis* pv. *vignicola* strains from worldwide origin on CCM medium compared to NGA medium

Xav[a] strains	Plating efficiency (%)[b,c]
1b2	61.3[d]
4	89.8
12a2	134.7
16g	89.6
19bg	79.2
23a	163.5
28a1g	188.6
30a1	78.0
35a3	100.1
39a4	144.3
43b2	156.7
44b1	45.6
51b3	82.9
56a	99.3
59a	106.6
FNPB1	99.2
536a1g	173.2
NCPPB 3187	100.0
267-5	122.2
267-10	77.1
268-10	111.1
317-2	103.0
Mean	109.4

[a]*Xav = Xanthomonas axonopodis* pv. v*ignicola*.
[b]Plating efficiency % = ((CFU/Petri dish on CCM medium)/(CFU/Petri dish on NGA)) × 100.
[c]NGA: nutrient broth (8 g), glucose monohydrate (11 g), yeast extract (3 g) and agar (14 g) per l of demineralized water; on NGA 72 h, on CCM 96 h growth.
[d]Means of two replicates and four counts of agar plates.

dilution levels on CCM, whereas *X. axonopodis* pv. *vignicola* colonies were generally only detectable from the fourth and fifth dilution levels on NGA (Figure 1B), except in leaf sample 2 and seed samples, where the number of saprophytes was low. Colony sizes of the pathogen were 1.5 mm on CCM and 5 mm on NGA 96 h after incubation. Five types of saprophytes appeared generally on NGA, but only two types in a few cases on CCM. In platings from the stem sample from Benin on CCM, few flat, yellowish and whitish colonies of saprophytes were easily distinguishable from the bigger, whitish, round and raised colonies of *X. axonopodis* pv. *vignicola*. The pathogen was easily detected on CCM in seeds from cowpea genotype IAR-48 from Kano and Badura, northern Nigeria (6×10^4 and 2.3×10^2 CFU g^{-1} seeds, respectively), and genotype IT92KD-257-7 from Kano (northern Nigeria) and Ibadan (western Nigeria) (4.2×10^5, 3×10^4 CFU g^{-1}seeds) (data not shown).

Isolation of X. axonopodis *pv.* vignicola *from soil*

Among three soil samples from cowpea fields in Benin and Nigeria, the pathogen was isolated from native soil only from the Cotonou sample and only on CCM, but not on NGA (Table 6, Figure 1C). Supplementing native soils from Cotonou and Ina with a suspension of *X. axonopodis* pv. *vignicola* strain 16 g, the pathogen was isolated on CCM (recovery 89.1% and 90.9%, respectively, in dilution levels 10^{-5} and 10^{-6}) and could easily be identified from *all* dilution levels, and no or only low saprophytic growth occurred. On the contrary, the pathogen was overgrown at dilution levels three and four on NGA, and grew at the 5th and 6th dilution levels on NGA (recovery 58.2% and 61.8% from soil from Cotonou and Ina, respectively), where the supplemented pathogen inoculum was still high, with considerable numbers of saprophytes present. Five and three different types of saprophytes appeared on NGA and CCM, respectively, in isolations from native soils. Generally, saprophytes growing on CCM were yellowish, whitish or bluish, flat and smaller than 0.5 mm in diameter 96 h after incubation. Saprophytic growth on both media was higher from soil than from plant samples. There was no difference in colony morphology of the pathogen isolated from plant and soil samples.

Discussion

The new medium designated as CCM medium is the first semi-selective medium developed for detection of *X. axonopodis* pv. *vignicola*. CCM inhibited growth of most saprophytes and allowed easy isolation of the pathogen from leaves, stems, seeds and soil samples, including pathogen-spiked soil samples as well as naturally contaminated soil, where isolation on the non-selective standard medium NGA failed or was more difficult. The few saprophytes which still grew on CMM medium exhibited smaller size and different color of colonies compared to colonies of the pathogen. Plating efficiency of most of the *X. axonopodis* pv. *vigni-*

Table 4. Recovery of *X. axonopodis* pv. *vignicola* in mixed suspensions with three or four saprophytes and suppression of saprophytes on NGA and CCM medium

Xav[a] + saprophytes	NGA[b]		CCM		Promotion (+) or inhibition (−) on CCM[c] (%)
	CFU[d]	Colony Size[e]	CFU	Colony size	
Xav 16g[f]	264	3.0	328	1.2	+24.2
P. fluorescens	148	5.0	88	1.0	−40.5
SP2	68	0.4	0	–	−100.0
SP3	152	3.0	140	0.2	−6.7
Xav 49a	360	5.0	460	1.5	+27.8
P. fluorescens	180	5.0	160	1.0	−11.1
SP2	128	0.5	0	–	−100.0
SP3	184	3.0	20	0.2	−89.9
Xav 16g	0	–	260	1.3	+100.0
Four saprophytes[g]	344	–	0	–	−100.0
Xav 49a	0	–	440	1.0	+100.0
Four saprophytes	320	–	0	–	−100.0

[a]*Xav* = *Xanthomonas axonopodis* pv. *vignicola*.
[b]NGA: nutrient broth (8 g), glucose monohydrate (11 g), yeast extract (3 g) and agar (14 g) per l of demineralized water; on NGA 72 h, on CCM 96 h growth.
[c]Promotion or inhibition in % based on CFU.
[d]Mean colony forming units (CFU) of two Petri dishes at dilution level 10^{-4}.
[e]Colony size in mm.
[f]Pathogen and saprophytes in suspensions of OD 0.06 at 660 nm each were mixed at 1:1:1:1 (:1 in case of four saprophytes).
[g]Four saprophytes: SP1, SP4, *Bacillus subtilis* and *Pantoea agglomerans*.

cola strains was higher or equal on CCM than on the standard NGA medium.

D-cellobiose was the optimal carbon source, and NH_4Cl selected as nitrogen-source. These components were also among the best carbon and nitrogen sources, respectively, for the growth of *X. axonopodis* pv. *manihotis* in a previous study (Fessehaie et al., 1999). D,L-methionine enhanced growth of *X. axonopodis* pv. *vignicola*. The semi-selective medium for *X. campestris* pv. *campestris* developed by Chun and Alvarez (1983) also contained methionine as growth factor which supported growth of the pathogen more than glutamic acid. In CCM, boric acid along with D-cellobiose suppressed growth of *P. fluorescens* and two saprophytes isolated from cowpea leaves. Boron in form of $Na_2B_4O_7$ was also used in a trace elements solution by Chun and Alvarez (1983) supplemented to their semi-selective medium. Colony size correlated to good growth in colony numbers and was therefore suitable for evaluation of bacterial growth.

Among the antibiotics, cefazoline, known to inhibit Gram-negative bacteria (Karyone et al., 1970), suppressed growth of most of the saprophytes with no or little effect on pathogen strains. Some variation of *X. axonopodis* pv. *vignicola* strains in growth of pure cultures on CCM medium was observed. A considerable diversity of *X. axonopodis* pv. *vignicola* strains in physiological and genetic characteristics was described (Verdier et al., 1998; Khatri-Chhetri et al., 2003). Cefazoline and lincomycin also allowed growth of 34 *X. axonopodis* pv. *manihotis* strains and one *X. cassavae* strain on a semi-selective medium developed for *X. axonopodis* pv. *manihotis* (Fessehaie et al., 1999).

The whitish and less slimy appearance of the *X. axonopodis* pv. *vignicola* colonies on CCM medium compared to yellow, slimy colonies on NGA medium may be due to the lack of bromine in the medium that is necessary for xanthomonadin production (Andrewes et al., 1973), and to suppression of slime production by some constituents of the CCM medium, respectively. Most of the xanthomonads produce extracellular polysaccharides on media containing glucose (Schaad and Stall, 1988). Batur (1994) found colonies of some *X. hortorum* pv. *pelargonii* strains to be yellow on

Figure 1. (A) Growth of *X. axonopodis* pv. *vignicola* strain 16g mixed with four saprophytes (SP1 and SP4 from cowpea leaves and *B. subtilis* and *P. agglomerans*) at equal cell densities on NGA[a] (left) and CCM medium (right). (B) Isolation of *X. axonopodis* pv. *vignicola* from a diseased cowpea stem on NGA (left) and CCM medium (right). (C) Plates of soil dilution for isolation of *X. axonopodis* pv. *vignicola* from a cowpea field in Benin on NGA (upper row) and CCM (lower row).

NGA, XTS (Schaad and Forster, 1985) and MSP (Mohan and Schaad, 1987) media, while colonies of other *X. hortorum* pv. *pelargonii* strains were reddish on KM-1-medium (Kim, 1982), cream-coloured on MXP-medium (Claflin et al., 1987), dark brown on ET-medium (Norman and Alvarez, 1989) and whitish on BSCAA medium (Randhawa and Schaad, 1984). Bromine was omitted from the medium because the whitish appearance of *X. axonopodis* pv. *vignicola* allowed to differentiate the pathogen from yellow-coloured saprophytes such as SP3 isolated from leaves, and saprophytes found in naturally infected stems from Benin and in soil.

Table 5. Isolation of *X. axonopodis* pv. *vignicola* strains from naturally infected cowpea plants and seeds on CCM and NGA media

Dilution	Leaf[a] Nigeria			Leaf Benin			Stem Nigeria			Stem Benin			Seeds Nigeria			Seeds Benin		
	NGA[b]		CCM	NGA		CCM	NGA		CCM	NGA		CCM	NGA		CCM	NGA		CCM
	Xav[c]	sap.	*Xav*	*Xav*	sap.	*Xav*	*Xav*	sap.	*Xav*	*Xav*	sap.	*Xav*	*Xav*	sap.	*Xav*	*Xav*	sap.	*Xav*
10^{-1}	0[d]	+++	+++	0	+++	+++	–	–	–	–	–	–	+++	+	+++	+++	++	+++
10^{-2}	0	+++	+++	+++	+	+++	0	++	+++	0	+++	+++	++	+	++	83	+	142
10^{-3}	0	++	++	++	0	++	0	++	++	0	+++	++	85	0	82	12	+	13
10^{-4}	++	14	++	++	0	++	++	64	++	0	+++	++	–	–	–	–	–	–
10^{-5}	–	–	–	–	–	–	20	17	31	30	27	94	–	–	–	–	–	–

[a]Leaf and stem samples from field-grown cowpea plants with bacterial blight symptoms from Nigeria and Benin. Seeds from naturally infected cowpea plants from genotypes IT92KD-257-7 and IAR-48 from Kano, Nigeria.
[b]NGA: nutrient broth (8 g), glucose monohydrate (11 g), yeast extract (3 g) and agar (14 g) per l of demineralized water; on NGA 72 h, on CCM 96h growth.
[c]*Xav* = *Xanthomonas axonopodis* pv. *vignicola;* sap. = non-identified, naturally occurring saprophytes.
[d]Colony forming units (CFU) per agar plate from 10-fold serial dilutions of plant macerates, 48 h after incubation on NGA, 96 h after incubation on CCM; 0 = no growth, +++ = uncountable number of colonies, ++ = many, + = few colonies, flowing together; colonies were counted where possible; – = not tested.

Table 6. Isolation of *X. axonopodis* pv. *vignicola* from native and pathogen-spiked soil samples from cowpea fields from Benin (Cotonou, Ina) and Nigeria (Ibadan) on CCM medium and NGA

Soil samples[a]	Bacteria	CFU/g soil[b]	
		NGA[c]	CCM
Cotonou	*Xav*	0	$2.5 \times 10^5 \pm 0$
	Saprophytes	1.0×10^{7}[d]	$1.4 \times 10^6 \pm 1.0 \times 10^5$
Ibadan	*Xav*	0	0
	Saprophytes	$9.0 \times 10^6 \pm 1.0 \times 10^6$	3.0×10^{6}[d]
Ina	*Xav*	0	0
	Saprophytes	$2.5 \times 10^7 \pm 5.0 \times 10^6$	4.0×10^{4}[d]
Cotonou + *Xav*[e]	*Xav*	$6.4 \times 10^7 \pm 3.5 \times 10^6$	$9.8 \times 10^7 \pm 1.2 \times 10^7$
	Saprophytes	$1.6 \times 10^7 \pm 9.5 \times 10^6$	$1.1 \times 10^5 \pm 1.5 \times 10^4$
Ina + *Xav*	*Xav*	$6.8 \times 10^7 \pm 2.2 \times 10^7$	$1.0 \times 10^8 \pm 2.8 \times 10^7$
	Saprophytes	$1.4 \times 10^7 \pm 6.0 \times 10^6$	0b
Control	*Xav*[f]	$1.1 \times 10^8 \pm 3.6 \times 10^7$	$9.9 \times 10^7 \pm 3.1 \times 10^7$

[a]Native and *Xav* – spiked soil samples from Cotonou and Ina, Benin and Ibadan, Nigeria.
[b]NGA: nutrient broth (8 g), glucose monohydrate (11 g), yeast extract (3 g) and agar (14 g) per l of demineralized water; on NGA 72 h, on CCM 96 h growth.
[c]Means and standard error of two replicates. For spiked samples, colony counts were made at the 10^{-5} and 10^{-6} dilution levels; at lower dilutions *Xav* could not be detected on NGA, while on CCM *Xav* was too numerous to count.
[d]No standard error due to flowing together of colonies in one replicate.
[e]*Xav* = *Xanthomonas axonopodis* pv. *vignicola* strain 16g added (0.5 ml of bacterial suspension of OD 0.06 at 660 nm supplemented to 1 g soil in 4.5 ml 0.01 M $MgSO_4$).
[f]CFU in 0.5 ml corresponding to 0.5 ml of bacterial suspension added to soil.

CCM medium inhibited growth of *P. agglomerans*, *B. subtilis* and three saprophytes isolated from cowpea leaves. *Pseudomonas fluorescens* and another, unidentified saprophyte, which could not be completely inhibited by CCM, could be distinguished from *X. axonopodis* pv. *vignicola* by their smaller size and different color of colonies. Growth of *X. axonopodis* pv. *vignicola* strains was strongly inhibited on NGA, when they were grown together with saprophytes, or when isolated from plant or soil samples. Recovery of the pathogen from spiked soil samples in higher dilution levels was also possible on NGA due to the high concentration of pathogen added, while in lower dilution levels, the pathogen was overgrown by saprophytes. Low recovery on NGA may also be due to antimicrobial substances with inhibiting or suppressing activity, which some saprophytes may release. Also Batur (1994) observed that growth of *X. hortorum* pv. *pelargonii* was inhibited on NGA when the pathogen was grown with various saprophytes.

In conclusion, the newly developed CCM medium is useful for isolation of *X. axonopodis* pv. *vignicola*, especially from plant and soil samples with low pathogen concentrations and/or when many epiphytic or saprophytic bacteria are present.

Acknowledgement

This study was funded by the Federal Ministry for Economic Cooperation and Development (BMZ), Germany.

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