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Semi-Selective Culture Medium for *Xanthomonas axonopodis* pv. *malvacearum* Detection in Cotton Seeds (*Gossypium hirsutum* L.)

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Abstract: The cotton disease known as angular leaf spot, caused by *Xanthomonas axonopodis* pv. *malvacearum* (Xam) has been causing cotton losses in several producing regions around the world. Xam is transmitted by seeds, which may be infected both externally and internally. Infected seeds constitute the main long-distance dissemination mode of the pathogen. In view of this, the use of healthy seeds is a must. To accomplish that, detection methodologies for the bacteria must be developed be used in seed health analysis laboratories. This study aimed to develop a semi-selective medium for Xam detection in cotton seeds. The semi-selective culture medium was named MSSXAN and it was consisted of peptone (5.0 g), beef extract (3 g), sucrose (5 g), soluble starch (10 g), agar (15 g), $CaCl_2$ (0.25 g), Tween 80 (10 mL), distilled water (1,000 mL), crystal violet solution at 1% (150 μL), cephalexin (50 mg^{l}*), methyl thyophanate (10 mg*) and chlorothalonil (10 mg*) - *added after culture medium autoclaving. This MSSXAN medium shows low repressiveness to Xam and it be used for isolation of this bacteria in cotton seeds health analysis.

Key words: Angular spot, bacterium, *Malvaceae*, seed pathology, phytopathogen

INTRODUCTION

There is a continuous quest for seed quality evaluation alternatives, particularly with regard to seed health. Seed health issues are increasingly important in international seed trade. With the advent of free trade, many countries are redefining their phytosanitary requirements with the goal of preventing introduction of a devastating pathogen into their country (Maddox, 1998). Currently, there are many methods available in plant pathology that can detect the presence of pathogens of various etiologies; nevertheless, there are few that can be used routinely in seed pathology laboratories. In this context, studies on methodologies designed to achieve better detection performance, quantification and identification of the pathogen present in seeds are justified, being indispensable to reduce the incidence of economically important diseases, thus preventing widespread epidemics and losses.

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It is crucial that researches be directed toward the development of methods that offer high sensitivity and reliability, together with fast results, at a lower cost.

It is consensus in the scientific community involved with cotton seed pathology that the extraction of bacteria by the method that uses maceration of seeds in liquids can be affected by contaminant microorganisms that make difficult to identify the pathogen. It must be borne in mind that the sensitivity of any method decreases as the incidence of saprophytic bacteria increases (Sheppard *et al*., 1989; León *et al*., 2006). Semi-selective culture media are considered rational alternatives for the detection of phytobacteria in seeds and have been developed by Moura and Romeiro (1993), Maringoni *et al*. (1994), Oliveira (1995), Metha and Bolognini (2003) and Frare (2006).

Semi-selective culture media are invaluable instruments for epidemiology and etiology studies in phytobacteriology and are also extremely useful to isolate phytopathogenic bacteria from plant tissues, soil and water (Klement *et al*., 1990).

The quest for excellence in a culture medium to detect bacteria in seeds relies on the standardization and validation of all tests performed, as well as on the selection of antibiotics. The latter must be easy to handle, inexpensive and readily available in the agrochemical or pharmaceutical market.

A method for Xam detection in cotton seeds is needed that possesses the following traits: easy to prepare, inexpensive, high detection sensitivity and reproducibility of results between laboratories. Considering the necessity for the development of a practical, effective and low-cost method for the routine analysis of cotton seeds in Brazil, this study was carried out with the objective of developing a semi-selective medium to detect Xam to serve as a viable alternative for the isolation and identification of this bacterium in seed health analysis laboratories.

MATERIALS AND METHODS

This study was performed at the Laboratory of Bacteriology, Department of Crop Production, Division of Plant Protection, Faculty of Agricultural Sciences, São Paulo State University, UNESP, Botucatu, São Paulo State, Brazil, from April 2004 to September 2005.

Antibiotics and Fungicides Sensitivity

To accomplish that, different antibiotics were tested in qualitative antibiograms, by the indirect disk diffusion plates method (or disk method), also inspired by the methodology of Sherwood *et al*. (1944), with modifications, on the growth of five Xam isolates. The technique consisted in homogenizing a Xam bacterial suspension in autoclaved NSA (nutrient-sucrose-agar) medium at melting temperature (±45°C) and then pouring the mixture onto Petri dishes. Next, paper disks impregnated with antibiotic (Oxoid®) were distributed over the culture medium, which was incubated at 28°C for 24 h. A completely randomized experimental design was used, with five replicates for each Xam isolate. The evaluation consisted in checking (and quantifying) for the presence of a Xam growth inhibition halo around the disks (http//www.neels.org).

Based on the selection of antibiotics to which the target bacterium (Xam) was resistant, their inhibiting concentration on Xam growth was tested using quantitative antibiograms, that is, indirect determinations, using the antibiotic dilutions method in the culture medium. The antibiotics cephalexin, cefadroxil, lincomycin, nitrofurantoin and oxacillin were used at concentrations of 0, 5, 10, 15, 20, 30, 50 and 100 μg mL^{-1}, in qualitative antibiograms.

Next, the highest concentration of antibiotic that did not cause reduction in growth of Xam colonies was selected and tested in different previously isolated bacteria that occurred together with the cotton seeds. The bacteria were suspended in distilled and sterilized water and distributed over the culture medium containing antibiotic. The antibiotic was considered effective when it inhibited the growth of bacteria other than Xam, in cotton seeds.

The action of fungicides chlorothalonyl and methyl thiophanate with some selectect antibiotics on Xam and others bacteria from cotton seeds growth was evaluated. Chlorothalonil and methyl thiophanate was evaluated because these fungicides were used on semi-selective culture media for isolation of *Xanthomonas axonopodis* pv. *phaseoli* (Maringoni *et al*., 1994) and *Curtobacterium flccumfaciens* pv. *flaccumfaciens* (Maringoni *et al*., 2006) on bean seeds and they inhibit micelial growth of several fungi associated with seeds.

Growth of Xam Isolates on Semi-Selective Culture Medium and Nutrient-Sucrose-Agar

The semi-selective medium consisted of: peptone (5.0 g), beef extract (3.0 g), sucrose (5.0 g), soluble starch (10.0 g), agar (15 g), $CaCl_2$ (0.25 g), Tween 80 (10.0 mL), distilled water (1,000 mL), crystal violet solution at 1% (150.0 μL), cephalexin (50.0 mg*), methyl thyophanate (10.0 mg*) and chlorothalonil (10.0 mg*)- *added after culture medium autoclaving. The composition of Nutrient-Sucrose-Agar (NSA) is: peptone (5.0 g), beef extract (3.0 g), sucrose (5.0 g) and agar (15 g) and distilled water (1,000 mL). Ten Xam isolates were checked and 100 μL of the bacterial dilution (10^{-6} to 10^{-9}) were plated onto the culture media (semi-selective and nutrient-sucrose-agar) in each Petri dish plate (5 plates), spread across the surface with a Drigalski loop and followed incubation at 28°C, for 72 h. The evaluation consisted of counts of the numbers of Xam colonies that grew on the culture medium after that period.

Detection of Xam in Cotton Seed Lots

Twenty five lots of cotton seeds, produced in 2004 in the mid-west region of Brazil, were evaluated for presence of Xam. For each seed sample, five sub-samples of 100 g (seeds without linter) or 120 g (seeds with linter) were obtained and each one was submitted to maceration in 300 mL (seeds without linter) or 500 mL (seeds with linter) of distilled and sterilized water, at 5°C, for 18-24 h. The resulting suspensions of seed maceration were sowed, by striae, or centrifuged (0, 1 L of suspension centrifuged at 480,000 rotations per hour during 1/6 h). Then, they were ressuspended in 1 mL of distilled and sterilized water and sowed in the semi-selective culture medium. The Petri dishes were incubated at 28-30°C, during 72-96 h and the colonies that came with cultural characteristics similar to the growth of a pattern pure isolated of Xam (circular, yellow, mucoide and bright colonies, presence of hydrolysis of starch and lipolysis of Tween-80 in the culture medium) were observed in the dishes. The seed lot was considered infected when there was growth of one or more bacterial colonies with characteristics similar to pattern isolate of Xam, in at least one of the five sub-samples analyzed.

Characterization of Similar Isolates to Xam Obtained from Seeds

Nineteen bacterial isolates obtained from the seed lots, with similar characteristics to Xam and a typical isolate of Xam were submitted to biochemical, physiological, morphological, serological and pathogenic characterization. The biochemical, physiological and morphological tests were: cellular morphology, differential coloration of Gram, solubility in KOH, growth in YDC culture medium, hydrolysis of esculin, starch, casein and gelatin,

catalase, reduction of nitrite to nitrate, growth at 40ºC and lipolysis of Tween-80, accomplished. The serological test was indirect immunofluorescence, with polyclonal antiserum against Xam, as methodology described by Dezordi (2006). The pathogenicity tests were accomplished in cotton plants (cultivate Acala 90), using the inoculation method by infiltration of the leaf blade, according to Klement *et al*. (1990).

RESULTS AND DISCUSSION

The qualitative antibiogram revealed that Xam *in vitro* sensitivity to antibiotics was variable among the various isolates of this bacterium, that is, some isolates had a resistant behavior, some were sensitive and others yet were intermediate with regard to the action of antibiotics. The sensitivity reaction among Xam isolates in the presence of antibiotics was different when the following were used: ticarcillin, erythromycin, cefaclor, penicillin G, clindamycin, cefixime, bacitracin, streptomycin, sulfatrim, fosfomycin, levofloxacin, cefetamet, cefadroxil, neomycin, amoxicillin, nitrofurazone, sulfazotrim, cefodizime and teicoplanin (Table 1).

Table 1: Growth inhibition halo and sensitivity or resistance reaction of five *Xanthomonas axonopodis* pv. *malvacearum* isolates to different antibiotics and concentrations, in a gel diffusion bioassay

		Xanthomonas axonopodis pv. *malvacearum* isolates									
		12.400		12.471		12.392		12.430		12.397	
Antibiotic	Conc. (µg disk^{-1})	Halo	Reaction	Halo	Reaction	Halo	Reaction	Halo	Reaction	Halo	Reaction[1]
		(mm)									
Clavulanic acid +amoxicillin	20	26.5	S	35.0	S	30.0	S	26.5	S	35.0	S
Pipemidic acid	2	25.0	S	31.0	S	22.0	S	20.0	S	30.0	S
Nalidixic acid	30	26.0	S	28.0	S	25.1	S	25.5	S	25.1	S
Amikacin	30	27.2	S	24.2	S	27.3	S	23.9	S	30.6	S
Amoxicillin	20	15.0	R	22.0	S	20.5	S	20.0	I	25.0	S
Ampicillin	30	22.9	S	25.2	S	22.0	S	18.7	S	24.4	S
Azithromycin	15	32.5	S	34.1	S	31.8	S	33.4	S	29.7	S
Aztreonam	30	31.0	S	27.9	S	26.9	S	27.3	S	30.6	S
Bacitracin	10	12.3	I	20.2	S	17.9	S	13.2	S	16.5	S
Carbenicillin	100	32.0	S	28.8	S	27.4	S	27.4	S	33.2	S
Cefaclor	30	20.6	S	6.6	R	21.0	S	3.0	R	21.8	S
Cefadroxil	30	15.0	I	0.0	R	0.0	R	0.0	R	15.0	I
Cephalexin	30	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
Cefazolin	30	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
Cefepime	30	38.5	S	31.9	S	37.9	S	37.4	S	36.4	S
Cefetamet	10	25.0	S	0.0	R	0.0	R	15.0	I	13.0	R
Cefixime	5	20.0	S	22.2	S	20.2	S	23.8	S	18.4	I
Cefodizime	15	30.0	S	22.0	S	25.0	S	15.0	I	35.0	S
Cefuroxime	30	27.2	S	21.8	S	26.8	S	25.0	S	28.0	S
Cefotaxime	30	26.5	S	29.0	S	23.0	I	23.5	S	33.5	S
Cefoxitin	30	27.6	S	26.6	S	28.2	S	30.6	S	31.2	S
Ceftazidime		31.2	S	32.2	S	30.8	S	30.0	S	31.1	S
Cephalothin	30	23.7	S	10.0	R	15.8	I	3.6	R	24.2	S
Ciprofloxacin	5	33.3	S	33.5	S	31.0	S	35.1	S	31.6	S
Clarithromycin	15	24.9	S	30.4	S	25.0	S	25.2	S	24.8	S
Clindamycin	2	10.6	R	8.2	R	9.7	R	17.3	I	13.6	R
Doxycycline	30	32.0	S	40.0	S	31.0	S	35.0	S	32.5	S
Erythromycin	15	24.6	S	19.6	I	25.6	S	21.5	S	29.2	S
Spiramycin	10	15.0	S	28.0	S	24.5	S	20.0	S	25.0	S
Streptomycin	30	21.5	S	32.0	S	22.5	S	24.0	S	32.0	S
Furazolidone	20	28.0	S	25.0	S	24.0	S	37.5	S	21.5	S
Fosfomycin	200	17.0	S	6.0	R	8.0	R	10.5	R	0.0	R

Table 1: Continued

		Xanthomonas axonopodis pv. *malvacearum* isolates									
		12.400		12.471		12.392		12.430		12.397	
Antibiotic	Conc. (µg disk^{-1})	Halo	Reaction	Halo	Reaction	Halo	Reaction	Halo	Reaction	Halo	Reaction[1]
		(mm)									
Gentamicin	120	20.5	S	19.9	S	21.6	S	20.0	S	22.5	S
Imipenem	10	35.0	S	30.0	S	40.0	S	40.0	S	42.5	S
Kanamycin	30	26.0	S	32.5	S	32.0	S	25.5	S	40.0	S
Kanamycin	30	26.3	S	25.5	S	24.0	S	23.2	S	25.0	S
Levofloxacin	5	20.0	I	30.0	S	35.0	S	25.0	S	39.0	S
Lincomycin	2	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
Methicillin	5	17.0	S	16.4	S	15.0	S	17.4	S	17.2	S
Mupirocin	30	8.0	R	0.0	R	8.8	R	3.0	R	11.3	I
Neomycin	30	19.0	S	20.0	S	23.5	S	15.0	I	25.0	S
Netilmicin	30	19.0	S	30.0	S	26.5	S	19.5	S	30.5	S
Netilmicin	30	20.2	S	20.4	S	20.4	S	22.1	S	24.2	S
Nitrofurantoin	300	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
Nitrofurazone	15	18.5	S	10.0	R	16.0	I	18.0	S	12.0	R
Novobiocin	30	9.5	R	15.0	R	13.0	R	7.0	R	10.5	R
Ofloxacin	5	28.6	S	30.2	S	33.6	S	35.8	S	31.3	S
Oxacillin	1	0.0	R	2.8	R	0.0	R	2.1	R	0.0	R
Pefloxacin	5	30.0	S	29.2	S	28.3	S	33.0	S	30.2	S
Penicillin G	10	19.9	I	22.2	I	13.8	R	22.5	I	20.0	I
Polymyxin B	300	17.6	S	14.4	S	18.2	S	16.8	S	18.1	S
Streptomycin	100	20.5	S	21.4	S	18.3	S	19.7	S	17.2	I
Sulbactam+ ampicillin	10	31.0	S	35.0	S	25.0	S	32.0	S	35.0	S
Sulfatrim	250	20.0	S	20.0	S	13.0	I	18.5	S	15.0	I
Sulfazotrim	15	20.0	I	25.0	S	25.0	S	17.5	I	30.0	S
Sulfonamide	250	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
Sulfamethoxazole	23	30.0	S	25.8	S	25.6	S	25.0	S	27.2	S
Teicoplanin	30	10.0	I	0.0	R	11.0	I	0.0	R	15.0	S
Tetracycline	30	25.0	S	35.0	S	30.0	S	35.0	S	35.0	S
Thiamphenicol	30	27.5	S	35.0	S	35.0	S	27.5	S	35.0	S
Ticarcillin+ clavulanic acid	75	26.5	S	35.0	S	38.5	S	35.0	S	40.0	S
Ticarcillin	10	24.2	S	20.4	I	24.4	S	22.5	I	25.7	S
Tobramycin	10	19.0	S	25.0	S	19.5	S	17.5	S	19.0	S
Trimethoprim	23	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
Vancomycin	30	25.0	S	22.6	S	24.7	S	26.0	S	24.1	S

S: Sensitive; R: Resistant; I: Intermediate, [1]Average of five replicates of each isolate

This suggests that, in nature, different Xam isolates undergo variations that result in variability among isolates of the same species, causing them to react distinctly to the same antibiotics. According to Romeiro *et al*. (1997), this behavior could be related to the type of constitutive multiple resistance to antibiotics possessed by the bacteria. Padilha and Costa (2002) verified that resistance to antibiotics in many microorganisms occurs due to presence of gene-bearing plasmids, which code for the synthesis of enzymes that inactivate specific antibiotics. These are termed R (resistance) or R factor plasmids. Isolates with the R factor could be mutants that exhibit different tolerances to drugs such as antibiotics and chemotherapics, in other words, these are isolates that at some point suffered alterations in their chemical or physical DNA structure.

The Xam isolates showed resistance to 21 different antibiotics, that is, there was no formation of a Xam growth inhibition halo around the antibiotic disk. Xam was naturally resistant to the following antibiotics: lincomycin, nitrofurantoin, oxacillin, cephalexin, cefadroxil, mupirocin, cephalotin, cefaclor, penicillin G, clindamycin, cefixime, novobiocin, fosfomycin, cefadroxil, amoxicillin, nitrofurazone, cefazolin, trimethoprim, sulfonamide,

Table 2: Growth of five *Xanthomonas axonopodis* pv. *malvacearum* isolates at different concentrations of antibiotics

		Isolates				
Antibiotics	Concentration (µg mL^{-1})	12.400	12.471	12.392	12.430	12.397
Nitrofurantoin	0	+	+	+	+	+
	5	+	+	+	+	+
	10	+	+	+	+	+
	15	+	+	+	+	+
	20	+	+	+	+	+
	30	+	+	+	+	+
	50	+	+	+	+	+
	100	+	±	±	±	±
Lincomycin	0	+	+	+	+	+
	5	+	+	+	+	+
	10	+	+	+	+	+
	15	+	+	+	+	+
	20	+	+	+	+	+
	30	+	+	+	+	+
	50	+	+	+	+	+
	100	+	+	±	+	+
Oxacillin	0	+	+	+	+	+
	5	±	+	±	+	±
	10	±	±	±	±	-
	15	±	-	±	±	-
	20	±	-	±	-	-
	30	-	-	-	-	-
	50	-	-	-	-	-
	100	-	-	-	-	-
Cephalexin	0	+	+	+	+	+
	5	+	+	+	+	+
	10	+	+	+	+	+
	15	+	+	+	+	+
	20	+	+	+	+	+
	30	+	+	+	+	+
	50	+	+	+	+	+
	100	+	+	+	+	+
Cefadroxil	0	+	+	+	+	+
	5	+	+	+	+	+
	10	+	+	+	+	+
	15	+	+	+	+	+
	20	+	+	+	+	+
	30	+	+	+	+	+
	50	+	+	+	+	+
	100	±	+	±	+	+

+: Colonies similar to the control treatment; ±: Partial growth inhibition, -: Growth inhibition

cefetamet and teicoplanin, at the concentrations cited. The antibiotics that had characteristics such as low price, liquid or powder formulation and product availability in the Brazilian market were: cephalexin, cefadroxil, lincomycin, oxacillin and nitrofurantoin. These were submitted to quantitative antibiogram tests to Xam, to verify their potential to be used in the preparation of a semi-selective culture medium.

The quantitative antibiogram evaluation results can be shown in Table 2. *In vitro* Xam sensitivity occurred at oxacillin concentrations higher than 10 µg mL^{-1} and not sensitivity to nitrofurantoin, lincomycim, cephalexin and cefadroxil at 5 to 100 µg mL^{-1}.

It was observed that cephalexin allowed Xam to grow in the NSA culture medium at all concentrations evaluated (0 to 100 µg L^{-1}); therefore, this is an interesting antibiotic to be added to semi-selective media. Concentrations 50% lower than the highest concentrations at which most isolates proved resistant were selected, that is, the culture medium was prepared with 50 µg L^{-1} cephalexin. This result is in agreement with Di *et al*. (1991), who

Table 3: *In vitro* action of different antibiotics and fungicides at different concentrations, added to the nutrient-sucrose-agar culture medium, on the growth of *Xanthomonas axonopodis* pv. *malvacearum* (Xam) and saprophytic bacteria isolated from cotton seeds

		Bacterial inhibition (%)	
Treatments	Concentration (μg mL^{-1})	Saprophytic[1]	Xam[2]
Cefadroxil	50	50	20
Cefadroxil	100	50	50
Cefadroxil+cephalexin	50+50	75	0
Cephalexin	50	75	0
Cephalexin	100	75	25
Cephalexin+methyl thiophanate+chlorothalonil	50+10+10	75	0
Nitrofurantoin+lincomycin+oxacillin+methyl thiophanate +chlorothalonil	50+50+1+10+10	8.3	0
Nitrofurantoin+lincomycin+oxacillin+methyl thiophanate +chlorothalonil	100+100+1+10+10	16.7	40
Control	0	0.0	0

[1]Twelve isolates, [2]Five isolates

studied *Xanthomonas* sp. selectivity in rice seeds and observed that the antibiotic cephalexin did not cause any reduction in growth to isolates of this bacterium and Maringoni *et al*. (1994) used this antibiotic on semi-selective medium for isolation of *X. axonopodis* pv. *phaseoli* from bean seeds.

Suppression results can be shown in Table 3. The incorporation of cephalexin at a 50 μg mL^{-1} concentration, plus the fungicides methyl thyophanate and chlorothalonil at concentrations of 10 μg mL^{-1} each, into the NSA culture medium, provided a 75% control of bacterial saprophytic isolates and no growth repression of Xam isolates. According to Randhawa and Schaad (1983), the addition of antibiotic and antifungal compounds to the basic medium to inhibit saprophytes must be meticulously studied, since the least degree of sensitivity to these compounds can be detrimental to the recovery of the target bacteria from the seeds. According to Klement *et al*. (1990), selective and semi-selective culture media are generally suppressive, even to the organism for which it was developed.

The joint incorporation of cephalexin (50 μg mL^{-1}) and chlorothalonil (10 μg mL^{-1}) plus methyl thyophanate (10 μg mL^{-1}) into the NSA culture medium provided satisfactory suppression (75%) of the various saprophytic isolates, without restraining Xam growth (Table 3). According to Valarini (1995), the use of cephalexin (40 mg) and chlorothalonil (50 mg) to detect *Xanthomonas campestris* pv. *vesicatoria* in tomato seeds is an effective method in view of its excellent specificity and sensitivity to the pathogen. Maringoni *et al*. (1994) used cephalexin, chlorothalonil, benomyl, nalidixic acid and nitrofuratoin on semi-selective culture medium for isolating *X. axonopois* pv. *phaseoli* from bean seeds.

Although the devised semi-selective medium does not completely suppress growth of the bacterial flora, non-controlled saprophytic isolates formed whitish bacterial colonies, making it easier to discriminate Xam colonies because of their yellow color. According to Claflin and Raimundo (1987), it is hard to develop a culture medium that can restrict the growth of all contaminants among the diversified microflora that is normally found associated with seeds.

Starch, crystal violet (1%) and Tween-80 were added to the culture medium in an attempt to facilitate the identification of suspected Xam colonies. This is in agreement with Klement *et al*. (1990), who stated that selectivity is normally achieved with the use of specific carbon sources, antibiotics, or other inhibiting compounds that favor the growth and identification of the pathogen in culture medium.

It was observed that when starch hydrolysis by Xam occurred, there was the formation of a discolored, lighter and clear halo surrounding the bacterial colonies growth, indicating that the isolates were capable of producing amylase. This trait is helpful in the identification of suspected Xam colonies. A similar result was found by Maringoni *et al*. (1994), who added starch to a semi-selective culture medium and verified hydrolysis of starch around the bacterial colonies; this trait helped to discriminate *X. axonopodis* pv. *phaseoli*. According to Frare (2006), incorporation of starch into the semi-selective medium is also an option when the expected selectivity of the antibiotic in relation to non-target bacteria cannot be obtained, since the addition of iodine allows the discrimination of amylolytic from non-amylolytic bacterial isolates.

The incorporation of crystal violet dye into the NSA culture medium did not decrease Xam growth, which allows it to be used in the preparation of the semi-selective culture medium. The incorporation of 150 µl L^{-1} crystal violet solution at 1% into the basic medium was useful to stain the medium, therefore improving the visualization of starch hydrolysis by the bacteria. This result agrees with a study by Ackermann (1977), who reported crystal violet bacterial action only against Gram-positive bacteria. According to Neder (1992), several dyes cause alterations or even inhibit the growth of some bacteria, at specific concentrations. Oliveira (1995) added 50 mg L^{-1} of crystal violet to Kado 523 medium and observed a significant color change in colonies of *X. campestris* pv. *vesicatoria*.

Lipolysis of Tween-80 by Xam was observed in the prepared culture medium, after incubation for 48 h at 28°C. The reactions were considered positive when the formation of opaque, whitish halos occurred around the suspected Xam colonies. These results are in agreement with Mcguire *et al*. (1986), who used a Tween-80 medium to detect *X. vesicatoria* pv. *vesicatoria* in tomato seeds.

Xam showed growth in both the semi-selective and the non-selective culture media (Table 4). Only the isolate 6 of Xam presented smaller amount of colonies in the semi-selective medium when compared to the number of colonies developed in the nutrient-sucrose-agar medium.

The semi-selective culture medium prepared in this study also proved promising to detect Xam in cotton seeds naturally infected. It was observed that Xam was present in 19 of the 25 cotton seed samples evaluated (Table 5).

After incubation for 72-96 h in the semi-selective medium, several suspected Xam colonies appeared (with a mucoide, convex, raised aspect and a bright, yellow color, with smooth edges, hydrolysis of starch and lipolisys of Tween-80). The pathogenicity test allowed the identification of suspected Xam colonies, isolated from seeds in the semi-selective culture medium to be confirmed, due to the manifestation of angular leaf spot in the inoculated cotton leaves (Table 6).

Table 4: No. of unit colony-forming of *Xanthomonas axonopodis* pv. *malvacearum* isolates in semi-selective culture medium and in nutrient-sucrose-agar

	UFC ($mL^{-1}\times10^{9}$)	
Isolates	Semi-selective medium	Nutrient-sucrose-agar
1	1.01	1.29
2	1.01	1.20
3	1.05	1.01
4	0.83	0.86
5	1.01	1.10
6	0.60	1.07
7	0.77	0.54
8	0.01	0.01
9	0.01	0.01
10	0.01^{9}	0.01

Average of five replicates for each isolates

Table 5: Detection of *Xanthomonas axonopodis* pv. *malvacearum* in macerates of cotton seeds samples with or without centrifugation, using a semi-selective culture medium

	Cotton seed sub samples									
	A		B		C		D		E	
Seed sample	C[1]	N[2]	C	N	C	N	C	N	C	N
1	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+
13	-	-	-	-	-	-	-	-	-	-
14	+	+	+	+	+	+	+	+	+	+
15	-	-	-	-	-	-	-	-	-	-
16	+	-	+	-	+	-	+	-	+	-
17	+	+	+	+	+	+	+	+	+	+
18	+	+	+	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+
21	+	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+	+

[1]C: Centrifugation; [2]N: No centrifugation; +: Presence of colonies like Xam; -: Absence of colonies like Xam

Table 6: Morphological, physiological, biochemistry, sorological and pathogenic characteristics of nineteen isolates with similar characteristics to *Xanthomonas axonopodis* pv. *malvacearum* originated from cotton seeds

	Bacterial isolate																			
Test	Xam[1]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Gram-negative stick	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KOH	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Reduction of nitrate to nitrite	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Yelloy, convex and mucoide colonies in YDC medium	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of esculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of casein	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lipolysis of Tween 80	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 40°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pathogenicity in cotton leaves cv. Acala 90	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Imunoinfluorescence	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[1]*Xanthomonas axonopodis* pv. *malvacearum*, +: Positive reaction, -: Negative reaction

It was observed that, no matter whether the maceration liquid from naturally or artificially infected cotton seeds was centrifuged or not, the presence of Xam could be verified using the semi-selective medium (Table 5). According to Moura and Romeiro (1993), many variables cause higher or lower bacterial concentrations within a seed lot and among lots. High detection sensitivity toward the isolation of a target pathogen is a chief characteristic in the

preparation of semi-selective media. Since, the primary sources of Xam inoculation in Xam-free areas consist of infested seeds, even low infestation rates must be detected.

The incidental appearance of contaminant bacteria in the semi-selective medium during incubation of plates containing semi-selective medium did not disturb detection of the target pathogen, since those were easily identified due to auxiliary identification traits, such as a lack of lipolysis of Tween-80; lack of hydrolysis of starch around the colonies and whitish color of most contaminant colonies that appeared onto the medium.

All the 19 bacterial isolates obtained from seeds presented morphological, physiological, biochemistry, cultural, serological and pathogenic characteristics similar to the pattern isolate of Xam and they presented serological positive reaction for the antiserum against Xam (Table 6). This way, they were identified as Xam.

According to Klement *et al*. (1990), Maringoni *et al*. (1994), Metha and Bolognini (2003) and Frare (2006), the successful use of semi-selective or selective culture media to detect plant pathogens in seeds occurs when aspects related to sensitivity, specificity, precision, relative ruggedness, interpretation and prediction of results are taken into consideration, as well as their adaptability to the laboratory routine, for instance, simplicity, quickness, standardization and low cost, in addition to components easily purchased in the market. Therefore, the final composition of the semi-selective medium for Xam (MSSXAM) detection was as follows: peptone (5.0 g), beef extract (3.0 g), sucrose (5.0 g), soluble starch (10.0 g), agar (15 g), $CaCl_2$ (0.25 g), Tween 80 (10.0 mL*), distilled water (1,000 mL), crystal violet solution at 1% (150.0 μL), cephalexin (50.0 mg*), methyl thyophanate (10.0 mg*) and chlorothalonil (10.0 mg*)- *added after culture medium autoclaving.

CONCLUSION

The culture medium developed, composed of 3.0 g of meat extract, 5.0 g of peptone, 15.0 g of agar, 5.0 g of sucrose, 1000 mL of distilled water, 0.25 g of $CaCl_2$, 10.0 g of soluble starch, 10.0 mL of Tween 80, 150 μL of crystal violet solution at 1%, 50 mg cephalexin, 10 mg of methyl thiophanate and 10 mg chlorothalonyl, was semi-selective and effective in isolation and detection of *X. axonopodis* pv. *malvacearum* in naturally infected cotton seed. The use of this semi-selective culture medium for Xam detection on a routine basis in seed health analysis laboratories is an alternative for the diagnosis of this bacterium in cotton seeds, in order to prevent the occurrence of angular leaf spot epidemics in the cotton industry.

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