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An Effective and Low-Cost Culture Medium for Isolation and Growth of *Xylella fastidiosa* from Citrus and Coffee Plants

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Abstract. Buffered charcoal–yeast extract medium (BCYE) has been used for isolation of *Xylella fastidiosa* from citrus (*Citrus sinensis*) and coffee (*Coffea arabica*) plants affected by citrus variegated chlorosis (CVC) and coffee leaf scorch (CLS). BCYE is composed of ACES (2-[2-amino-2oxoethyl) amino]-ethanesulfonic acid) buffer, activated charcoal, yeast extract, L-cysteine, ferric pyrophosphate, and agar. ACES buffer is costly and not always commercially available in Brazil, and the L-cysteine and ferric pyrophosphate need to be filter sterilized in 0.22-μm pore membranes before inclusion in the medium. Omission of L-cysteine, addition of magnesium sulfate, and replacements of ACES and ferric pyrophosphate for potassium phosphate and ferrous sulfate resulted in an effective, less expensive, and entirely autoclavable medium, named phosphate buffered charcoal-yeast extract medium (PCYE). The final cost of PCYE was approximately one tenth that of BCYE. Its effectiveness was tested for the isolation of *X. fastidiosa* from symptomatic leaves collected from 52 citrus plants affected by CVC and 43 coffee plants affected by CLS. PCYE was as effective as BCYE and has been used routinely in our and other laboratories for isolation, growth, and quantification of *X. fastidiosa* from plant tissues.

In Brazil, *Xylella fastidiosa* causes citrus variegated chlorosis (CVC), plum leaf scald (PLS), and coffee leaf scorch (CLS) [1–3]. The pathogen also was detected by polymerase chain reaction (PCR) in asymptomatic weeds growing in citrus groves [4]. In the field, the pathogen is transmitted from diseased to healthy trees by several species of sharpshooters, and disease control demands applications of insecticides to decrease vector populations, pruning and removal of symptomatic trees to decrease inoculum sources, and planting of healthy young trees [5].

Since the description of *X. fastidiosa* as the causal agent of PLS, CLS, and mainly of CVC, several studies focusing on disease epidemiology, vector transmission, and plant–pathogen interactions have been conducted for implementing disease-management practices. In most studies, PCR was used for pathogen detection in plant tissues. Although of high specificity and sensitivity, regular PCR is not appropriate to answer questions that involve quantification of bacterial cells growing inside the plant. The traditional isolation and culture on agar plates allows not only pathogen quantification but also its recovery for further physiologic and pathologic investigations.

The fastidious and slow-growing nature of *X. fastidiosa* required the development of nutritionally complex culture media [6–8]. These media contain components, not well defined chemically, that are appropriate for the isolation and culture of apparently all *X. fastidiosa* strains. The most used are periwinkle wilt (PW) broth or agar medium [6] and buffered charcoal–yeast extract agar (BCYE) [8]. Not all components of these media are always commercially available in Brazil. Importation takes time and is expensive making their routine use in many research laboratories prohibitive. To solve this problem, we performed modifications in BCYE. The final version, named phosphate buffered charcoal-yeast extract medium (PCYE) was as effective, less expensive, and easier to prepare than BCYE.

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Material and Methods

Modifications of BCYE agar medium. BCYE agar medium was prepared according to Wells et al. [8], except that the agar was substituted by Gelrite-Gellan Gum (Sigma, St. Louis, MP), as described for the PW medium [9]. BCYE is composed of 10.0 g ACES (2-[2-amino-2oxoethyl) amino]-ethanesulfonic acid) buffer, 2.0 g activated charcoal, 10.0 g yeast extract, 0.4 g L-cysteine $HCL.H_2O$, 0.25 g ferric pyrophosphate, and 8.0 g Gelrite in one liter of water. L-cysteine and ferric pyrophosphate were filter sterilized (0.22-µm pore size) and added to the autoclaved components and equilibrated at 50°C. The pH of the final solid medium was 6.9 at 25°C. In contrast, 1 L of the final version of PCYE contained 1.0 g KH_2PO_4, 1.1 g K_2HPO_4, 0.25 g $FeSO_4.7H_2O$ (Vetec Química, Duque de Caxias, RJ), 0.4 g Mg $SO_4.7H_2O$, 2.0 g activated charcoal, 10.0 g yeast extract, and 8.0 g Gelrite or 10.0 g agar. L-cysteine was omitted [10]. The pH was adjusted to 6.9 with 1 M potassium hydroxide solution (KOH) before autoclaving.

***X. fastidiosa* isolation and culture on PCYE.** The modified medium was tested using symptomatic leaves from 52 citrus (*Citrus sinensis*) and 43 coffee (*Coffea arabica*) plants. The citrus leaves were collected from 9 plants of 8-year-old "Pêra Rio" growing in the field (Gavião Peixoto, SP) or from 43 1-year old greenhouse-grown "Valencia" artificially inoculated with the 9a5c strain, the same used for genome sequencing. The coffee leaves were collected from 14 plants of 10-year old Catuaí cultivar growing in the field (Ribeirão Preto, SP). Each sample consisted of 50 ul 10^2 to 10^5 serially diluted phosphate-buffered saline macerate of petioles and midribs of 2 to 3 leaves surface sterilized as described [11]. The macerate of each sample was distributed with a Drigalsky flamed glass wire on the surface of 3 80 mm-diameter disposable Petri dishes containing 30 ml BCYE or PCYE medium. The plates were sealed with parafilm and incubated at 28°C for 30 days, at which time colonies were counted and diameters measured with a digital paquimeter (Digimess, Shiko Precision Gaging Ltd., China). In total, the diameter of 137 colonies of field citrus samples (82 on BCYE and 55 on PCYE) and 67 of coffee field samples (37 on BCYE and 30 on PCYE) were measured. The identity of the recovered colonies was assessed by PCR using the *X. fastidiosa* specific primers 272-1 and 272-2 [12]. Individual colonies were removed, transferred to 100 ul autoclaved Milli Q water, boiled for 10 minutes, and aliquots transferred to PCR tubes containing 25 ul PCR master mix. PCR running conditions were as described [12]. The SAS system (GLM procedure) and *F* test were used for data analysis and comparisons.

Results and Discussion

PCYE was as effective as BCYE medium for the primary isolation of *X. fastidiosa* from citrus trees, allowing pathogen growth from all nine samples. Media comparison for the isolation of *X. fastidiosa* from samples of citrus seedlings and coffee trees could not be done completely because of the presence of contaminants in five PCYE plates containing leaf macerate of citrus seedlings and in one BCYE and four PCYE plates containing leaf macerate of coffee trees. Not considering the contaminated samples, higher frequencies of positive isolations were obtained for PCYE for the isolation of *X. fastidiosa* from citrus seedlings (36 of 38 versus 40 of 43 in BCYE) and coffee trees (35 of 39 versus 33 of 42 in BCYE). The level of agreement of both media was 86.8% for citrus seedlings, 100.0% for citrus trees, and 87.2% for coffee trees (Table 1). The level of disagreement for pathogen isolation from citrus seedlings was 13.2%, with two (5.3%) BCYE+/PCYE–negative and three (7.9%) BCYE-/PCYE–positive samples, and 12.8% from coffee trees, with five BCYE-/PCYE–positive samples.

Table 1. Agreement of BCYE and PCYE media in the isolation of *X. fastidiosa* from leaf midribs of artificially inoculated citrus seedlings or of naturally infected citrus and coffee trees.

Culture medium and positive or negative isolation	Sample of greenhouses, citrus seedings (%)	Sample of field citrus trees (%)	Sample of field coffee trees (%)
BCYE+/PCYE+	33 (86.8)	9 (100)	30 (76.9)
BCYE+/PCYE–	2 (5.3)	0	0
BCYE–/PCYE+	3 (7.9)	0	5 (12.8)
BCYE–/PCYE–	0	0	4 (10.3)

PCYE allowed growth of significant higher numbers of *X. fastidiosa* cells from samples of citrus seedlings and coffee trees (Table 2). The pathogen could be detected on both media as small whitish colonies readily visible with the naked eye 12 to 15 days after plating. Although not periodically quantified, the growth rate seemed to be similar for the CVC and CLS strains of *X. fastidiosa* in both media. Thirty days after plating, the colonies reached average diameters of 2.16 mm (range 1.52 to 2.79) and 2.04 mm (range 1.44 to 2.86) in BCYE and PCYE for the CVC strain and 2.09 mm (range 1.39 to 2.67) and 2.14 mm (range 1.82 to 3.03) in BCYE and PCYE for the CLS strain. Lysate PCR confirmed the identity of the isolated colonies as *X. fastidiosa* with an amplified DNA fragment of approximately 500 bp.

X. fastidiosa is considered a fastidious plant pathogen requiring nutritionally complex culture media for isolation and growth. Those most commonly used are PW and BCYE, which contain a group of substances such as bovine serum albumin (BSA) or activated charcoal-both of which favor *X. fastidosa* growth, apparently by removing compounds present in plant tissues that are toxic to the microorganism [6, 8]-and ferric pyrophosphate and L-cysteine in PW as nutrient source. BCYE also includes the organic ACES buffer. ACES needs to be dissolved in KOH solution, and BSA, L-cysteine, and ferric pyrophosphate need to be filter sterilized through 0.22-µm pore membrane before addition to the autoclaved components. These procedures are tedious and time consuming and make PW and BCYE media prone to contamination during preparation. Also, some of their components, particularly BSA and ACES, are costly and not always available for purchase. In fact, the BSA

Table 2. Average number of colony forming units per plate of *Xylella fastidiosa* recovered from leaf midribs of symptomatic citrus seedlings artificially inoculated and growing under greenhouse conditions or of naturally infected citrus and coffee trees growing in the field, thirty days after plating.

Culture medium	Greenhouse infected citrus seedlings	Field infected citrus trees	Field infected coffee trees
BCYE	14.465[a]	66.478[a]	13.974[a]
PCYE	18.316[b]	79.870[a]	23.171[b]
F test	10.33**	1.93[NS]	10.05**
m. s. d. (Tukey, 5%)	2.6411	19.995	5.4396

component of PW medium has become almost unavailable lately because of importation restrictions of substances of bovine origin that could potentially be associated with the bovine spongiform encephalopathy disease.

The fastidious nature of *X. fastidiosa* has, however, been questioned lately with the demonstration that some strains can grow on media containing only yeast extract or beef extract and peptone as nutrient source [10, 13, 14]. Given this information and driven by the necessity of finding an alternative for the original BCYE for isolation and culture of the CVC and CLS strains of *X. fastidiosa*, changes were made in the BCYE agar medium. Deletion of L-cysteine [10] and replacement of the costly imported compounds for less expensive, locally available, and nonthermolabile substitutes resulted in a medium that was entirely autoclavable, that costs approximately one tenth of the original BCYE, and that is effective for isolation and culture of *X. fastidiosa*. Pinprick inoculations also determined that the colonies isolated in PCYE remain pathogenic to coffee, citrus and young tobacco seedlings [15] (manuscript in preparation). Thus far, only the CVC and CLS strains of *X. fastidiosa* have been tested on PCYE. Its efficacy for other strains must yet be determined.

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