Comparison of Immunofluorescence and Two Assays for Detection of *Xanthomonas campestris* pv. *undulosa* in Seeds of Small Grains

E. DUVEILLER, International Maize and Wheat Improvement Center (CIMMYT), Lisboa 27, Apdo Postal 6-641, Col. Juárez, Deleg. Cuauhtémoc, 06600 México D.F., Mexico, and C. BRAGARD, Unité de Phytopathologie, Université Catholique de Louvain, Place Croix du Sud 2 Bte 3, B-1348 Louvain-la-Neuve, Belgium

ABSTRACT

Duveiller, E., and Bragard, C. 1992. Comparison of immunofluorescence and two assays for detection of *Xanthomonas campestris* pv. *undulosa* in seeds of small grains. Plant Dis. 76:999-1003.

A monoclonal antibody specific for *Xanthomonas campestris* pathovars in the *translucens* group, including *X. c.* pv. *undulosa*, the causal agent of bacterial leaf streak or black chaff in wheat and triticale, was used in immunofluorescence and a dot-immunobinding assay to detect pathogens in aqueous seed extracts. These techniques were compared to dilution plating of seed wash water as potential routine seed-indexing methods. Twenty-six cereal seed lots were harvested from plants with different levels of natural disease incidence grown in three locations in Mexico. Among the 26 lots analyzed, pathogen-free seed came from clean fields principally in Ciudad Obregón, where conditions are less favorable for its development. Pathogen populations detected with dilution plate ranged from 1.3×10^4 to 5.3×10^6 cfu/g of air-dry weight. Populations were recovered from seed lots that had been stored for 3 yr. Some discrepancies in pathogen detection occurred among the different methods used. Seed lots with high pathogen populations were consistently identified with all three methods. Differences in detection thresholds were found with seed wash water as compared to reference strain from pure culture. Apparently a substantial number of dead cells were present in wash water from some seed lots. Immunofluorescence was more sensitive than dot-immunobinding assay and produced more reproducible results. It is proposed as a standard for indexing germ plasm. Because of the high variation in data, direct counts of fluorescent bacterial cells under UV microscopy will probably not be necessary for identifying infected seed. It is recommended that immunofluorescence-positive seed lots should not be considered for sowing in areas favorable for disease development.

Infected or infested seed is considered to be the major source of primary inoculum for bacterial leaf streak or black chaff in small grain cereals. The disease is a concern for international germ plasm exchange. It is caused in wheat and triticale by *Xanthomonas campestris* pv. *undulosa* (Smith, Jones and Reddy) Dye, in barley by *X. c.* pv. *translucens* (Jones, Johnson and Reddy) Dye, and in rye by *X. c.* pv. *secalis* (Reddy, Godkin and Johnson) Dye. Production of wheat and triticale is increasing in environments prone to the disease, including parts of Brazil, Paraguay, Argentina, Bolivia, Ethiopia, Uruguay, and Pakistan (13,15, 29). Seed transmission of the pathogen has been studied in wheat and barley (24,43,50); the bacterium has been isolated from triticale and rye seed (29). Because of the absence of effective chemical seed treatment, control of bacterial leaf streak or black chaff should be centered on seed certification (19,31).

At present, dilution plate analysis with a semiselective agar medium is the most common and reliable way of detecting *X. c. undulosa* in infected seed (13,14, 40,41). The method has the advantage of detecting living cells. However, its accuracy is limited by the selectivity of the medium, pathogenic colonies may be difficult to distinguish from nonpathogenic bacteria commonly found on cereal grains, and it is laborious when used for routine seed indexing. A rapid detection method involving 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside has been investigated (33,34), but the lack of specificity for *X. c. undulosa* does not allow its use in routine detection (E. Duveiller, *unpublished*). Mehta (29) proposed a simple seedling injection technique to detect contaminated cereal seed. Seed (20 g) are shaken thoroughly for 90 min in 20 ml of sterile saline water, the suspension is inoculated into 20-day-old seedlings with a hypodermic syringe, and *Xanthomonas* streak symptoms are assessed 7–12 days after inoculation. This method, although useful for quarantine purposes, is time-consuming and cannot be used to assess the amount of bacteria in a seed lot.

Serological methods (36,42,44) require less time than dilution plate analysis for detection of plant pathogenic bacteria and may offer the possibility of a rapid specific procedure for the testing of germ plasm. However, the older serology techniques employ antisera that are heterogeneous mixtures of polyclonal antibodies, are difficult to reproduce at a later date with a defined specificity, and may frequently fail to react with certain strains of a given pathogen species or cross-react with nontarget species. Unlike polyclonal antibodies, monoclonal antibodies (MAbs) have the advantage of being producible in unlimited quantities, with identical specificity, affinity, and immunoglobulin class (42). Recently, a dot-immunobinding assay (DIA) was developed for use with MAbs and other antibodies (8,22). Immunofluorescence (IF) has proved to be very sensitive for the detection of antibody-antigen complexes (46). It requires highly specific antiserum (37), but IF can be significantly improved by using MAbs (38).

Bacterial leaf streak of cereals has a worldwide distribution (13) but is sporadic and usually limited to specific environments, mostly in developing countries where resources may not justify the development of MAbs. A CIMMYT contract at Université Catholique de Louvain (UCL) in Belgium has been used to develop MAbs specific to *X. c. undulosa*. The purpose of this study was to use an MAb to index seed lots for *X. c. undulosa* and to evaluate IF and DIA in potential routine detection procedures to replace time-consuming dilution plate assays.

MATERIALS AND METHODS

Seed samples. Naturally infested seed lots were collected in Mexico from bread wheat (*Triticum aestivum* L.), durum wheat (*T. turgidum* var. *durum* Desf.), barley (*Hordeum vulgare* L.), triticale (× *Triticosecale* Wittmack), and rye (*Secale cereale* L.). Seed was harvested from areas where disease incidence varied, and consequently it was expected that differing populations of bacteria would be found. Twenty-six seed lots (Table 1) from different crops were harvested during 1987–1989 in three areas: CIANO (Centro de Investigacion Agricola del Noroeste) station near Ciudad Obregón (39 m above sea level [masl]), CODAGEM (Centro de Organización y Desarollo en el Estado de Mexico) and Atizapan experiment stations near Toluca (2,640 masl) and El Batan (2249 masl), where CIMMYT (Centro Internacional de Mejoramiento de Maiz y Trigo) headquarters are located. Ciudad Obregón, in northwestern Mexico (Sonora State), is characterized by a subtropical dry environment but crops are irrigated. The other stations are in humid-temperate,

Accepted for publication 17 March 1992.

subtropical highlands, where bacterial leaf streak is usually more severe than in other growing areas. The seed was collected in fields with different disease levels. Based on symptom incidence, except for lots 9, 15, and 24 (Table 1), fields were classified in four categories: 0 = clean (no symptoms), 1 = low disease incidence (<25%), 2 = moderate to severe disease incidence (25–75%), and 3 = very high disease incidence (76–100%). Lots 9, 15, and 24 were prepared with seed harvested on tillers showing <25, 25–49, and >49% severity on flag leaf and were considered in categories 1, 2, and 3, respectively. Each seed lot was tested three times (on 18 and 22 June and 4 July 1990) by three techniques (IF, DIA, and dilution plate) simultaneously with a 10-g sample per seed lot. *X. c. undulosa* reference strain CFBP3085 diluted to about 3×10^7 cfu/ml, according to turbidity measured with a spectrophotometer, was used as positive control.

Monoclonal antibody. Monoclonal antibody AB3-B6 prepared against the CFBP3085 reference strain from Mexico was produced at Centre d'Application des Anticorps Monoclonaux, Unité de Phytopathologie, UCL, Louvain-la-Neuve, Belgium. Splenocytes from immunized 'Lou/c' rats were fused with IR983F myeloma cells according to the procedure of Bazin (6). The monoclonality obtained by the limiting dilution technique was determined by the reaction of the hybridoma cell culture supernatant with monospecific anti-rat IgM serum (Experimental Immunology Unit [IMEX], UCL, Brussels). In indirect immunofluorescence and enzyme-linked immunosorbent assay (ELISA), AB3-B6 antibody reacted positively with all 44 strains of *X. campestris* tested. Based on host range tests, pathovars *cerealis, hordei, secalis, translucens*, and *undulosa* were represented. The MAb did not differentiate between these five pathovars. The reaction was negative with 11 strains of *X. campestris* pathovars or species not related with bacterial leaf streak. No positive reaction was found, either, with 36 non-*Xanthomonas* species, including the wheat pathogens *Clavibacter tritici* (Hutchinson) Davis et al, *Erwinia rhapontici* (Millard) Burkholder, and *Pseudomonas syringae* pv. *atrofaciens* (McCullough) Young et al, and saprophytic bacteria isolated from small grains.

Dilution plate test. Ten-gram seed samples were shaken (200 rpm) in 100 ml of sterile distilled water for 30 min at room temperature. A 0.5-ml sample of the liquid phase was diluted 10-fold in sterile saline (NaCl 0.85%) and plated (0.1 ml) onto a modified Wilbrink's medium (WBC) that included 0.75 g/L of boric acid and 10 mg/L of cephalexin (14). For each liter of WBC, 5 g of bactopeptone, 10 g of sucrose, 0.5 g of K_2HPO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of Na_2SO_4 anhydrous, and 15 g of agar were dissolved in 850 ml of distilled water, autoclaved, and then cooled to 45 C. Boric acid (0.75 g) that had been autoclaved separately in 150 ml of distilled water and cooled to 45 C was added to the medium. Then 10 mg of cephalexin (1 ml of a 10 mg/ml stock solution in 75% ethanol) and 75 mg of cycloheximide dissolved in 2 ml of 75% ethanol were mixed and the medium distributed to plates (14). Two samples of each dilution were dispensed to separate plates. Colonies were counted after the plates had been incubated for 4 days at 30 C. Colony-forming units per gram of air-dry weight seed were calculated. Representative colonies were cloned on WBC and tested for pathogenicity by wound inoculation of the stem of five leaf-stage seedlings (Alondra) at a point 5 cm above the soil level. The seedlings were held at 25 C for 6 days in a humid room and evaluated for symptoms of bacterial leaf streak.

Immunofluorescence test. In preliminary experiments, the use of saline and Tween 20 (Sigma) in the wash water led to an accumulation of Tween and salts on the slide and clumping of the bacteria on the glass. This clumping increased autofluorescence. Also, the reduction in

Table 1. Comparison of bacterial number, dot-immunobinding assay (DIA), and immunofluorescence microscopy (IF) from seed of small grains potentially infected by *Xanthomonas campestris* pv. *undulosa*

Lot	Crop	Genotype	Year	Field aspect[a]	Experiment 1			Experiment 2			Experiment 3		
					cfu/g[b]	DIA[c]	IF[d]	cfu/g	DIA	IF	cfu/g	DIA	IF
1	Barley	H272/Nopal's	1988	0	0	0	0	0	0	0	0	0	0
2	Barley	Trompillo	1988	0	0	0	0	0	0	0	0	0	0
3	Bread wheat	Alondra	1988	0	0	0	0	0	0	0	0	0	3
4	Durum wheat	Altar	1988	0	0	0	0	0	0	0	0	0	0
5	Durum wheat	Altar	1988	0	0	0	0	0	0	0	0	0	0
6	Durum wheat	Yavaros	1988	0	0	0	1	0	0	0	0	0	0
7	Rye	Prolific	1988	0	0	0	0	0	0	0	0	0	0
8	Bread wheat	Alondra	1988	1	0	0	2	5.90	1	2	5.00	2	3
9	Bread wheat	Alondra	1988	1	5.60	2	4	5.58	3	5	5.46	3	5
10	Bread wheat	Alondra	1989	1	4.48	0	2	4.48	1	1	0	0	0
11	Durum wheat	Altar	1988	1	0	0	3	4.70	0	4	5.28	1	4
12	Durum wheat	Yavaros	1988	1	4.11	0	0	0	0	2	0	0	4
13	Triticale	Buffalo	1989	1	4.54	0	1	5.04	3	3	5.40	2	3
14	Triticale	Buffalo	1988	1	6.32	2	4	6.34	3	4	5.30	3	3
15	Bread wheat	Alondra	1988	2	5.81	2	5	5.78	3	5	5.58	2	4
16	Durum wheat	Altar	1988	2	4.65	2	5	4.93	3	5	4.65	3	4
17	Durum wheat	Yavaros	1988	2	5.30	2	4	5.18	3	5	5.23	3	5
18	Rye	Prolific	1988	2	4.88	0	4	5.40	2	5	5.70	1	4
19	Triticale	Buffalo	1989	2	6.00	2	5	6.23	3	5	5.78	3	4
20	Triticale	Stier	1989	2	6.15	1	3	4.88	0	2	6.36	1	3
21	Triticale	Stier	1989	2	6.46	2	3	6.68	3	5	6.56	3	3
22	Bread wheat	Alondra	1987	3	0	0	0	0	0	0	4.26	2	4
23	Bread wheat	Alondra	1988	3	5.18	2	2	4.88	3	1	5.08	3	4
24	Bread wheat	Alondra	1988	3	5.18	2	4	5.77	3	5	5.43	3	5
25	Rye	Prolific	1988	3	4.81	1	4	5.36	3	5	5.54	3	5
26	Triticale	Stier	1989	3	6.58	2	4	6.63	3	4	6.72	3	5
Control					7.54	4	5	7.58	4	5	7.56	4	5

[a] 0 = No disease; 1 = <25% incidence, 2 = 25–75% incidence, and 3 = 76–100% incidence, except for lots 9, 15, 24, which were harvested from tillers with <25%, 25–49% and >49% flag leaf area damaged and were considered in categories 1, 2, and 3, respectively.
[b] cfu/g = Colony-forming units per gram of air-dried seed, based on dilution plate of seed wash water onto modified Wilbrink's agar medium.
[c] DIA results rated as 0 (no reaction) through 4 (an intense color reaction).
[d] IF results rated as 0 (no reaction), 1 = 1–4, 2 = 5–20, 3 = 21–100, 4 = 101–400, and 5 = >400 fluorescent cells observed on 20 microscope fields (magnification of 1,000×).

surface tension associated with the Tween 20 led to movement of the suspension over the plastic between wells during drying under airflow. Therefore, distilled water was used to wash seed.

Forty microliters of seed wash water was pipetted directly from the flask into a 6-mm well on a multiwindows slide (Biomérieux, Charbonnières-les-Bains, France) and then fixed with hot air from a hair dryer. The indirect immunofluorescent staining method was applied (21,35,42). Wells were exposed (for 60 min) to MAb AB3-B6 diluted 100 times in sterile phosphate-buffered saline (PBS; 8 g of NaCl, 2.7 g of $Na_2HPO_4 \cdot 12H_2O$, 0.4 g of $NaH_2PO_4 \cdot 2H_2O$, and 1 L of distilled water, pH 7.2). The wells were then rinsed with PBS and exposed to a mouse anti-rat MAb conjugated with fluorescein isothiocyanate MARM4-FITC (IMEX, UCL, Brussels) that reacts with the light chain of the rat IgM molecule. The wells were again rinsed twice with PBS, and then three drops of buffered glycerine (100 mg of diphenilamine in 10 ml of PBS, pH 9.6, and 90 ml of glycerol) were added.

Slides were examined under a Carl Zeiss (Model K 17 II Z C) microscope with a high-pressure mercury ultraviolet lamp HBO-50, a Carl Zeiss filter combination 10 (ref. 487710), and a magnification of 1,000×. At this magnification, 2,175 microscope fields (each 0.013 mm^2) cover the slide window where the 40 μl has been fixed. Twenty microscope fields were examined for IF-positive bacteria, and a rating was assigned according to the following scale: 0 = 0, 1 = 1–4, 2 = 5–20, 3 = 21–100, 4 = 101–400, and 5 = >400 cells.

Dot-immunobinding assay. A pure nitrocellulose membrane with 0.45-μm pore size (Bio-Rad Trans-Blot transfer medium), previously immersed in TRIS-buffered saline solution (TBS; 20 mM of Sigma 7-9, 500 mM of NaCl, pH 7.5) was secured in a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Richmond, CA). Wash water was pipetted (200 μl) directly from the flask to three wells of the apparatus to allow three readings per test. After 1 hr at atmospheric pressure, a vacuum was applied, and the membrane was washed with 200 μl of TTBS (TBS containing 0.05% Tween 20, pH 7.5) per well. The membrane was then immersed (200 μl per well) for 30 min in a blocking solution (TBS containing 1% bovine serum albumin). It was then incubated (for 60 min) with MAb AB3-B6 (200 μl per well), diluted 500 times in the blocking solution. After two washings with TTBS (200 μl per well, eliminated by vacuum), the membrane was incubated for 60 min in peroxidase-labeled MAb MARK-PO (IMEX, UCL, Brussels), diluted 500 times in the blocking solution. Membrane coated with *X. c. undulosa*-(AB3-B6)-(MARK-PO) was removed from the apparatus and exposed to a solution prepared as follows: TTBS (50 ml) was added to 30 mg of 4-chloro-1-naphtol dissolved in 10 ml of methanol. In positive tests a gray precipitate was observed some minutes after adding 50 μl of perhydrol 30% H_2O_2. The intensity of the gray color was assessed according to a five-level scale, from 0 for no reaction to 4 for an intense reaction.

Detection limit. A test was performed to determine the detection end points of both IF and DIA. A concentrated suspension of the reference strain (CFBP3085) was prepared from a fresh bacterial culture (24-hr) grown on WBC medium and diluted 10-fold in distilled water, as when the seed was washed. Each dilution was tested with IF and DIA, whereas the viable populations were determined by dilution plate analysis. The specificity of the MAb was verified again, by DIA analysis of *X. c. undulosa* strain CFBP3085 and turbid suspensions of 20 saprophytic bacterial strains that had been isolated from wheat in Mexico, and suspended in distilled water.

RESULTS

Detection limits of DIA and IF methods. The DIA detection end point was 10^6 cfu/ml. With immunofluorescence, a clear positive reaction (>10 cells per microscope field) was observed with an aqueous cell suspension containing 10^5 cfu/ml, and positively reacting cells were still easily detectable at 10^4 cfu/ml. In contrast, with 10^3 cfu/ml, few fluorescent cells were found in the 20 microscope fields observed.

Comparison of infection levels in the seed. Five of the 26 lots tested (1, 2, 4, 5, and 7) appeared to be free of the causal organism, since the pathogen was not detected by any of the three methods in three separate experiments (Table 1). These samples came from fields showing no disease, with three of them in CIANO, an area where disease incidence was low during 1987–1988. Seed lots 3, 6, 10, and 22 yielded negative results with the three methods at least once over the three attempts, evidence that *X. c. undulosa* was not distributed uniformly within certain seed lots. These seed lots came from fields showing no disease or from fields with a low disease incidence, except for lot 22. Seed lot 22 came from a plot with more than 75% disease incidence, but it had been stored for 3 yr before being assayed. It is possible that viability of *X. c. undulosa* had decreased in this lot. Lots 23–26 also had been harvested from severely diseased fields, but at a later date than 1987, and they consistently yielded higher cfu-per-gram figures (Table 1).

Dilution plate analyses were in some cases difficult to interpret because of numerous colonies of nontarget species. However, all pure cultures obtained from single presumptive colonies of *X. c. undulosa* were pathogenic on wheat. The pathogen population detected in the dilution plate technique was never below 1×10^4 cfu/g of seed and was higher in triticale compared with wheat, ranging from 3.5×10^4 to 5.3×10^6 cfu/g. In triticale, both cultivars (Stier and Buffalo) were similarly infected independent of the appearance of the field (Table 1).

With the exception of lot 12, all samples that yielded living cells in the dilution plate test were positive for IF (n = 49), but the 1–5 ratings given in the IF were not quantitatively related to the populations found with the dilution plate tests (Table 1). In contrast, samples that were negative for the pathogen in IF were never found to be positive in the DIA. Six samples (7.7%) were positive for IF but negative for dilution plate, evidence that these seed lots were infested with dead cells or that antagonism hampered the detection of living cells. The result observed for lot 12, where the dilution plate test was positive and the serology tests were negative, could be due to either a weak fixation of the antigenic cells onto the multiwindows slide in the IF test or to a possible misidentification of *X. c. undulosa* on the agar plate. Considering the number of fluorescent cells observed under the microscope for a 40-μl aliquot, and the cfu per gram determined with dilution plate, we estimated that the number of IF-reacting cells were greater than the number of living cells in the seed. This seems particularly true with lot 16 (experiments 1 and 2), for which the IF score was 5 and the viable population below 10^5 cfu/g (Table 1).

The intensity of the DIA was generally higher for seed harvested from highly diseased plots as compared with seed from plots that showed low disease (Table 1). All DIA-positive samples were also positive in the IF and dilution plate tests, but the cfu per gram recovered in the latter was always lower than that expected from the end point determined with the DIA with the reference culture.

Disagreement occurred between dilution plating and the serology methods (one positive, one negative), with averages of 12.7 and 18.1% disagreement for dilution plate versus IF or DIA, respectively. Therefore, IF and dilution plate were the methods most consistently resulting in the same conclusions.

DISCUSSION

Many methods used to detect bacterial pathogens have been developed for vegetable crops (1,2,12,27,28,37,39, 45,48), but few have been implemented for routine seed testing of small grain cereals (9,14,29,40). We investigated use of serological methods involving MAbs to replace the laborious dilution plate detection method in an effort to develop a rapid procedure to detect cereal seed lots contaminated with the bacterium

responsible for black chaff (24,43,50). The value of an immunodiagnostic method is specificity, sensitivity, and reproducibility (37). MAbs for *X. c. campestris* have been produced (3,49) that are quite specific to that bacterium and have been evaluated for detecting the pathogen in crucifer seed (4). Similarly, MAbs have been developed against *X. c. oryzae* (7,23) and used to detect the pathogen in artificially infested seed samples (7). The work reported here is the first attempt to use a MAb developed against *X. c. undulosa* for direct detection of the pathogen in naturally infested cereal seed. The tests of water used to wash seed are the most common way to apply serological techniques for the detection of seedborne pathogens in seed-screening tests (20).

We found that serological methods can be used to detect *X. c. undulosa* in seed wash water. Nevertheless, as reported for *Pseudomonas syringae* pv. *phaseolicola* (Burkholder) Young et al in bean seed (20,48), inconsistencies occur among results of the different detection methods.

Dilution plate analysis is likely to underestimate the population that is present, because of antagonism among bacteria recovered directly from seed lots on the plate. This observation could not be confirmed either by IF or DIA for lot 22, which was stored 3 yr before being analyzed. In a previous study, the duration of the washing negatively affected the recovery of *X. c. undulosa* on WBC agar (14). This could be caused by increased exposure of the pathogen to antagonistic bacteria, by toxic compounds released from the seed, or by prolonged osmotic imbalances. In the present study, distilled water instead of saline plus Tween was used to extract the bacteria from seed. The use of distilled water led to fewer problems with serological tests. However, improved efficiency for recovery of pathogenic xanthomonads was associated with saline as compared with distilled water (39).

The use of DIA may allow workers to detect and then to deal with seed lots that are heavily infested with *X. c. undulosa*. Claflin and Ramundo (8) used DIA with polyclonal antibodies for the detection of the bacterium in wheat seed and found that the assay was positive when the cell concentration was higher than 10^5 cfu/ml. We used a MAb and found a similar detection threshold. From the tests of pure cultures, however, we concluded that the DIA led consistently to an overestimation of the population of *X. c. undulosa* in the seed lot, at least as compared with populations determined through dilution plate analyses. This is evidence that certain cells have lost viability yet retain antigenicity. Similar indication was obtained in a DIA developed to detect *X. c.* pv. *holcicola* (Elliott) Dye in wash water of infected plants with rabbit polyclonal antibodies (26). The DIA method is simple and requires inexpensive equipment. Its disadvantages are that it is more time consuming than IF, it has a slightly lower reproducibility than dilution plate and IF, some wells may not percolate easily if the membrane is obstructed by seed debris, and false positive readings may occur from dust buildup on the membrane when seed is dirty and not heavily infested. DIA also uses a larger quantity of the antiserum, and it may also have some background problem if washing is not adequate (42).

Compared with dilution plate and DIA, IF is sensitive and relatively rapid. Seed-testing procedures generally involve the combination of IF followed by plating onto agar medium to confirm positive results (32,45,48). For routine identification of plant pathogenic bacteria, IF with a specific antiserum is probably the best test available (36), but inoculum quantification is difficult (27). Direct manual counting of bacterial cells is extremely tedious and not practical for a large number of samples. However, an automated seed detection system was recently implemented for estimating *Clavibacter michiganensis* subsp. *sepedonicus* (Spieckermann and Kotthoff) Davis et al labeled with MAbs in IF (11). Fluorescent cells are assumed to be randomly distributed, and the number of microscope fields to be examined may change depending on cell concentration (10). With a 1,000× magnification and the observation of 20 microscope fields, a concentration of 7.6×10^3 cells per milliliter is needed to declare a 40-μl sample of seed wash water positive at $P = 0.05$. The number of microscope fields to be observed is 163 with 10^3 cells per milliliter but only 13 with a 10^4 cells per milliliter. The variability observed in our results suggests that quantification is critical to the decision as to whether infested seed lots should be discarded. With 10-g seed samples, conclusions derived from IF and DIA are comparable with those from dilution plate analysis. In the *X. c. xyli*–sugarcane pathosystem, the total cell population predicted from IF outnumbered that from plate counts, particularly for higher concentrations of the bacterium; extreme differences between IF estimates and plate count were due to unusually low plate counts, and lack of fit may have depended on dead cells (10). Loss of viability of bacteria appears difficult to quantify and may depend on the seed lot, period of storage, storage environment, weather conditions during harvest that may favor saprophytic flora antagonistic to *X. c. undulosa*, and seed infestation by soil dust.

Each of the techniques used here may underestimate pathogen population as the exact location of *X. c. undulosa* in the seed is not known. Bacteria infecting the embryo may not be released during the extraction procedure. So, comparison of results of different extraction periods or with different samples of a same seed lot is difficult. The survival of the pathogen in wheat seed can exceed 63 mo (18), but Klykov (25), as reported in Neergaard (30), showed that with two seed lots infested with 1.26×10^7 and 8.7×10^5 cfu/g, respectively, the recovery was reduced by 93.1 and 78.9% after storage for only 6 mo, and that more than 99.5% was not detectable after 3 yr. With *X. c. phaseoli*, even low populations in bean seed may lead to severe yield losses, and a zero tolerance has been instituted by many seed certification agencies (27). In contrast, *X. c. undulosa* appears to have a low transmission rate; seed lots with a low level of contamination can be planted without the development of disease in the field (38). So, detection methods for *X. c. undulosa* do not have to be highly sensitive (17,38), and in positive seed lots the exact evaluation of the population size above the threshold for disease development is probably of secondary importance. Under Idaho conditions, populations in seed wash solution (125 g of seed per 125 ml of saline) $>= 1 \times 10^3$ cells per milliliter are needed for development of black chaff in the field (17,40).

The most beneficial method of rapidly detecting bacteria in numerous samples might be ELISA (5,42). Yet, the applications of this technique in phytobacteriology have often been limited as compared to immunofluorescence microscopy, because with ELISA the sensitivity is 10–100 times lower than that of IF (42,48). Moreover, specificity with ELISA for detecting bacteria in plant material may be lower, since cross-reactions with other sample particles cannot be distinguished from specific reactions, as can be done in IF microscopy on the base of cell morphology (42). Another limiting factor with ELISA is the variability with which bacteria adsorb to the Plexiglas plate (38). Approaches such as immunoisolation (47) may offer new possibilities for serological and dilution plate methods. However, the use of IF with MAbs to the closely related xanthomonads responsible for bacterial leaf streak of cereals (16) seems a workable way to implement a rapid seed-testing scheme for discarding most heavily contaminated seed lots from germ plasm exchanges. The sample size can be increased, particularly if foundation seed has to be tested, but in germ plasm exchanges the amount of seed available is often small, and 10-g seed samples are usual. When positively reacting bacteria are found in one of 20 microscope fields at a 1,000× magnification, the seed lot should not be planted in areas known to be favorable for bacterial leaf streak development, since pathogen concentra-

tion of 1×10^3 cells per gram of seed is likely to induce an epidemic in disease-prone conditions.

ACKNOWLEDGMENTS

This work was conducted as part of the Collaborative Research Network on Bacterial Diseases of Wheat, CIMMYT, funded by the Belgian Administration for Development Cooperation (BADC). We are indebted to M. Verhoyen for his open collaboration and to J. Robinson for reviewing the manuscript.

LITERATURE CITED

1. Akerman, A., Zutra, D., Volcani, Z., and Henis, Y. 1973. Application of an immunofluorescent technique for detecting *Corynebacterium michiganense* and estimating its extent in tomato seed lots. Phytoparasitica 1:128.
2. Allan, E., and Kelman, A. 1977. Immunofluorescent stain procedures for detection and identification of *Erwinia carotovora* var. *atroseptica*. Phytopathology 67:1305-1312.
3. Alvarez, A. M., Benedict, A. A., and Mizumoto, C. Y. 1985. Identification of xanthomonads and grouping of strains of *Xanthomonas campestris* pv. *campestris* with monoclonal antibodies. Phytopathology 75:722-728.
4. Alvarez, A. M., Benedict , A. A., Or, G., and Mizumoto, C. Y. 1987. Identification of xanthomonads from crucifer seeds with monoclonal antibodies. (Abstr.) Phytopathology 77:1725.
5. Alvarez, A. M., and Lou, K. 1985. Rapid identification of *Xanthomonas campestris* pv. *campestris* by ELISA. Plant Dis. 69:1082-1086.
6. Bazin, H. 1982. Production of rat monoclonal antibodies with the Lou rat secreting IR983F myeloma cell line. Pages 615-618 in: Protides of Biological Fluids. H. Peeters, ed. Pergamon Press, Oxford.
7. Benedict, A. A., Alvarez, A. M., Berestecky, J., Imanaka, W., Mizumoto, C. Y., Pollard, L. W., Mew, T. W., and Gonzalez, C. F. 1989. Pathovar-specific monoclonal antibodies for *Xanthomonas campestris* pv. *oryzae* and for *Xanthomonas campestris* pv. *oryzicola*. Phytopathology 79:322-328.
8. Claflin, L. E., and Ramundo, B. A. 1987. Evaluation of the dot-immunobinding assay for detecting phytopathogenic bacteria in wheat seeds. J. Seed Technol. 11:52-61.
9. Cunfer, B. M. 1987. Testing cereal seeds for bacterial pathogens. Pages 259-265 in: Seed Pathology. Vol. 2. Int. Adv. Course. Passo Fundo, RS, Brazil.
10. Davis, M. J. 1985. Direct-count techniques for enumerating *Clavibacter xyli* subsp. *xyli*, which causes ratoon stunting disease of sugarcane. Phytopathology 75:1226-1231.
11. De Boer, S. H., and Hall, J. W. 1988. An automated microscope system for estimating the population of *Corynebacterium sepedonicum* cells labelled with monoclonal antibodies in immunofluorescence. Can. J. Plant Pathol. 10:215-220.
12. De Boer, S. H., and Wieczorek, A. 1984. Production of monoclonal antibodies to *Corynebacterium sepedonicum*. Phytopathology 74:1431-1434.
13. Duveiller, E. 1989. Research on '*Xanthomonas translucens*' of wheat and triticale at CIMMYT. Bull. OEPP/EPPO Bull. 19:97-103.
14. Duveiller, E. 1990. Seed detection of *Xanthomonas campestris* pv. *undulosa* using a modification of Wilbrink's agar medium. Parasitica 46:3-17.
15. Duveiller, E., Bragard, C., and Maraite, H. 1991. Bacterial diseases of wheat in the warmer areas—Reality or myth. Pages 189-202 in: Proc. Wheat for the Nontraditional Warm Areas Int. Conf. UNDP/CIMMYT, D. Saunders, ed.
16. Fang, C. T., Allen, O. N., Riker, A. J., and Dickson, J. G. 1950. The pathogenic, physiological and serological reactions of the form species of *Xanthomonas translucens*. Phytopathology 40:44-64.
17. Forster, R. L., and Schaad, N. W. 1985. Tolerance levels of seedborne *Xanthomonas campestris* pv. *translucens*, the causal agent of black chaff of wheat. Pages 974-975 in: Proc. Int. Conf. Plant Pathog. Bact., 6th. E. L. Civerolo, A. Collmer, R. E. Davis, and A. G. Gillapsie, eds.
18. Forster, R. L., and Schaad, N. W. 1990. Longevity of *Xanthomonas campestris* pv. *translucens* in wheat seed under two storage conditions. Pages 329-331 in: Proc. Int. Conf. Plant Pathog. Bact., 7th.
19. Fourest, E., Rehms, L. D., Sands, D. C., Bjarko, M., and Lund, R. E. 1990. Eradication of *Xanthomonas campestris* pv. *translucens* from barley seed with dry heat treatments. Plant Dis. 74:816-818.
20. Franken, A. A. J. M., and Van Vuurde, J. W. L. 1990. Problems and new approaches in the use of serology for seedborne bacteria. Seed Sci. Technol. 18:415-426.
21. Hampton, R., Ball, E., and De Boer, S., eds. 1990. Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens. American Phytopathological Society, St Paul, MN. 396 pp.
22. Hawkes, R., Niday, E., and Gordon, J. 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. 119:142-147.
23. Jones, R. K., Barnes, L. W., Gonzalez, C. F., Leach, J. E., Alvarez, A. M., and Benedict, A. A. 1989. Identification of low-virulence strains of *Xanthomonas campestris* pv. *oryzae* from rice in the United States. Phytopathology 79:984-990.
24. Jones, L. R., Johnson A. G., and Reddy, C. S. 1917. Bacterial blight of barley. J. Agric. Res. 11:625-643.
25. Klykov, A. P. 1945. The viability of the causal agent of black bacteriosis in wheat seed. Mikrobiologiya 14:413-414.
26. Leach, J. E., Ramundo, B. A., Pearson, D. L., and Claflin, L. E. 1987. Dot-immunobinding assay for detecting *Xanthomonas campestris* pv. *holcicola* in sorghum. Plant Dis. 71:30-33.
27. Malin, E. M., Belden, E. L., and Roth, D. A. 1985. Evaluation of the radioimmunoassay, indirect enzyme-linked immunosorbent assay, and dot blot assay for the identification of *Xanthomonas campestris* pv. *phaseoli*. Can. J. Plant Pathol. 7:217-222.
28. Malin, E. M., Roth, D. A., and Belden, E. L. 1983. Indirect immunofluorescent staining for detection and identification of *Xanthomonas campestris* pv. *phaseoli* in naturally infected bean seed. Plant Dis. 67:645-647.
29. Mehta, Y. R. 1990. Management of *Xanthomonas campestris* pv. *undulosa* and *hordei* through cereal seed testing. Seed Sci. Technol. 18:467-476.
30. Neergaard, P. 1977. Seed Pathology. Vol. 1. Halsted Press, John Wiley & Sons, New York. 839 pp.
31. Ralph, W. 1977. Problems in testing and control of seedborne bacterial pathogens: A critical evaluation. Seed Sci. Technol. 5:735-752.
32. Rat, B. 1987. How to use bacteria detection methods in a seed pathology routine laboratory. Pages 253-258 in: Seed Pathology. Vol. 2. Int. Adv. Course. Passo Fundo, RS, Brazil.
33. Sands, D. C., Mizrak, G., and Hall, V. N. 1984. An indicator dye for *Xanthomonas campestris* pv. *translucens*. (Abstr.) Phytopathology 74:881.
34. Sands, D. C., Mizrak, G., Hall, V. N., Kim, H. K., Bockelman, H. E., and Golden, M. J. 1986. Seed transmitted bacterial diseases of cereals: Epidemiology and control. Arab J. Plant Prot. 4:127-125.
35. Schaad, N. W. 1978. Use of direct and indirect immunofluorescence tests for identification of *Xanthomonas campestris*. Phytopathology 68:249-252.
36. Schaad, N. W. 1979. Serological identification of plant pathogenic bacteria. Annu. Rev. Phytopathol. 17:123-147.
37. Schaad, N. W. 1982. Detection of seedborne bacterial plant pathogens. Plant Dis. 66:885-890.
38. Schaad, N. W. 1987. Use and limitations of methods to detect seedborne bacteria. Pages 324-332 in: Seed Pathology. Vol. 2. Int. Adv. Course. Passo Fundo, RS, Brazil.
39. Schaad, N. W., and Donaldson, R. C. 1980. Comparison of two methods for detection of *Xanthomonas campestris* in infected crucifer seeds. Seed Sci. Technol. 8:383-391.
40. Schaad, N. W., and Forster, R. L. 1985. A semiselective agar medium for isolating *Xanthomonas campestris* pv. *translucens* from wheat seeds. Phytopathology 75:260-263.
41. Schaad, N. W., and Forster, R. L. 1989. Detection of *Xanthomonas campestris* pv. *translucens* in wheat. Pages 41-44 in: Detection of Bacteria in Seed and Other Planting Material. A. W. Saettler, N. W. Schaad, and D. A. Roth, eds. American Phytopathological Society, St. Paul, MN.
42. Schaad, N. W., Süle, S., van Vuurde, J. W. L., Vruggink, H., Alvarez, A. M., Benedict, A. A., de Wael, L., and van Laere, O. 1990. Serology. Pages 153-190 in: Methods in Phytobacteriology. Z. Klement, K. Rudolph, and D. C. Sands, eds. Akad. Kiadó, Budapest.
43. Smith, E. F., Jones, L. R., and Reddy, C. S. 1919. The black chaff of wheat. Science 50:48.
44. Trigalet, A., Samson, R., and Coleno, A. 1978. Problems related to the use of serology in phytobacteriology. Pages 271-288 in: Proc. Int. Conf. Plant Pathog. Bact., 4th.
45. Van Vaerenbergh, J. P. C., and Chauveau, J. F. 1987. Detection of *Corynebacterium michiganense* in tomato seed lots. Bull. OEPP/EPPO Bull. 17:131-138.
46. Van Vuurde, J. W. L. 1985. Detecting seedborne bacteria by immunofluorescence. Pages 835-842 in: Proc. Int. Conf. Plant Pathog. Bact., 6th. E. L. Civerolo, A. Collmer, R. E. Davis, and A. G. Gillapsie, eds. Nijhoff, Dordrecht.
47. Van Vuurde, J. W. L. 1987. New approach in detecting phytopathogenic bacteria by combined immunoisolation and immunoidentification assays. Bull. OEPP/EPPO Bull. 17:139-148.
48. Van Vuurde, J. W. L., Van den Bovenkamp, G. W., and Birnbaum, Y. 1983. Immunofluorescence microscopy and enzyme-linked immunosorbent assay as potential routine tests for the detection of *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* in bean seed. Seed Sci. Technol. 11:547-559.
49. Yuen, G. Y., Alvarez, A. M., Benedict, A. A., and Trotter, K. J. 1987. Use of monoclonal antibodies to monitor the dissemination of *Xanthomonas campestris* pv. *campestris*. Phytopathology 77:366-370.
50. Wallin, J. R. 1946. Seed and seedling infection of barley, bromegrass, and wheat by *Xanthomonas translucens* var. *cerealis*. Phytopathology 36:446-457.