European Journal of Plant Pathology **109:** 407–417, 2003.

Mini review

The application of flow cytometry and fluorescent probe technology for detection and assessment of viability of plant pathogenic bacteria

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Accepted 31 October 2002

Key words: fluorescence, flow cytometry, fluorescent probes

Abstract

Conventional methods to detect and assess the viability of plant pathogenic bacteria are usually based on plating assays or serological techniques. Plating assays provide information about the number of viable cells, expressed as colony-forming units, but are time-consuming and laborious. Serological methods, such as the enzyme-linked immunosorbent assay (ELISA) and immunofluorescence microscopy (IF), can be performed in a shorter timespan than most plating assays, but they do not discriminate between live and dead cells. Flow cytometry (FCM) in combination with fluorescent probe technology is a rapid, sensitive, and quantitative technique to detect microorganisms and assess their viability. Quantitative information on the presence and viability of plant pathogenic microorganisms is valuable for risk assessment regarding disease transmission and disease development. FCM has been applied successfully in the fields of food microbiology, veterinary science, and medical research to detect and distinguish between viable and non-viable bacteria. The aim of this review is to show the potential of FCM and fluorescent probe technology for the field of plant pathology.

Introduction

The presence of harmful bacteria on plants is commonly determined by isolation and culturing of microorganisms on artificial growth media followed by identification. Depending on the bacterium, isolation and identification can be a very time-consuming process, taking from two days to up to one or more weeks. In spite of the use of semi-selective culture media, secondary microorganisms may overgrow or suppress growth of the target bacterium.

Immunological and molecular techniques are also routinely used for detection of bacteria. Both techniques can be performed in a shorter timespan than most plating assays. However, these techniques provide the user with only semi-quantitative information, which for various applications is not satisfactory. Moreover, neither technique distinguishes between viable and dead cells of the target organism, whereas such information is essential for effective decision-making, e.g. for allowing import and export or for treatment of seed lots. Both quantitative data and information about viability provide the basis for risk assessment regarding disease development.

This review will focus on new techniques for detection and assessment of viability of plant pathogenic bacteria, based on flow cytometry (FCM) and fluorescent probe technology.

Flow cytometry

Flow cytometry is a technology which enables the determination of physical and/or biochemical characteristics of biological particles, such as bacterial cells, in a suspension. The advantage of this technology is the

capacity for simultaneously measuring several parameters, based on light scattering and fluorescence, on thousands of individual cells within a few minutes. A schematic configuration of a typical flow cytometer is given in Figure 1. Cells in a fluid stream pass through a flow cell, where at the interrogation point a laser beam illuminates the cells and excites fluorescent compounds attached to or contained in the cells. These compounds may be fluorescent dyes which have been added to the suspension or autofluorescent molecules naturally occurring in the cells.

Several parameters can be measured simultaneously. Forward scatter (FS) is the amount of laser light scattered at narrow angles from the axis of the laser beam,

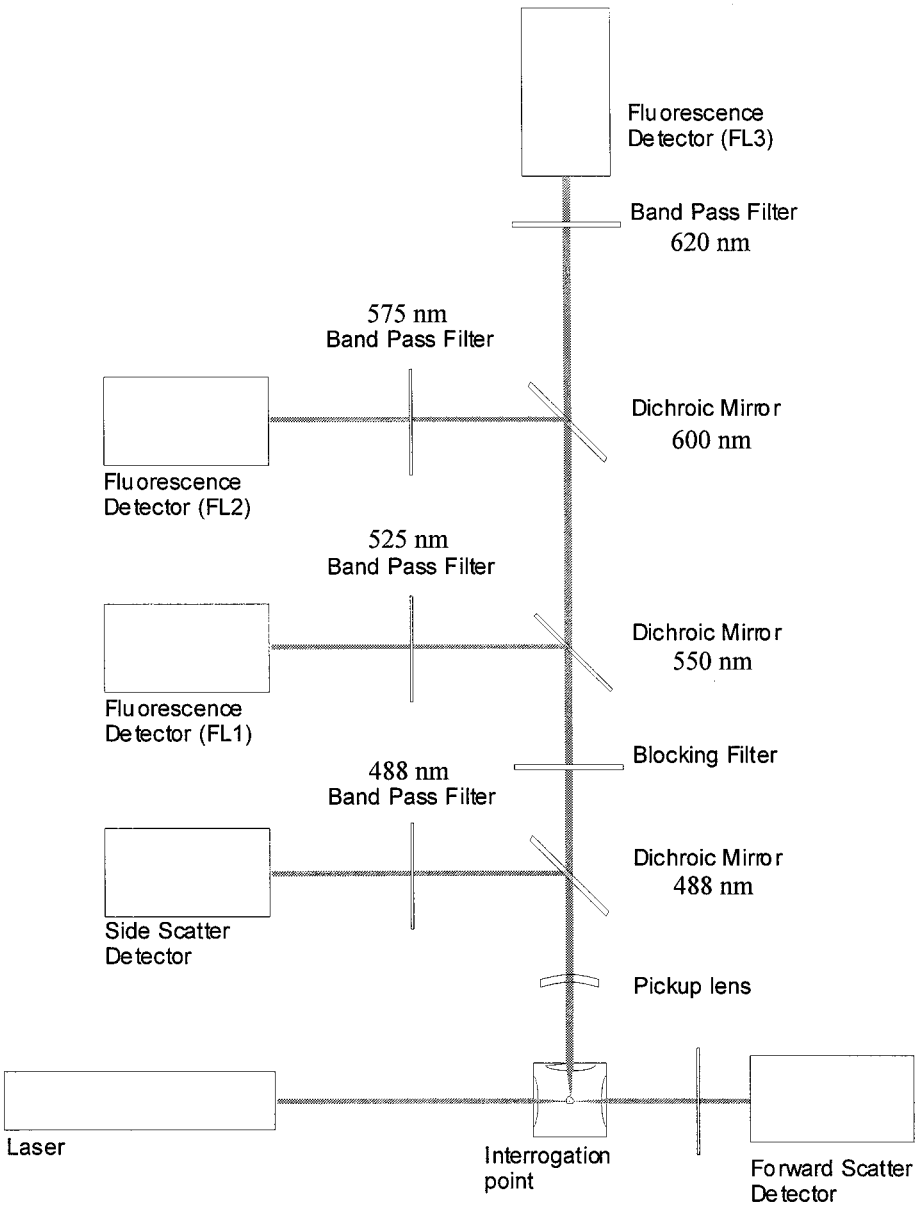

Figure 1. Schematic representation of a typical flow cytometer showing filter configuration, forward and side scatter detectors and fluorescence detectors. The green, orange, and red fluorescence are measured by the FL1, FL2, and FL3 detector, respectively.

and correlates with the relative cell size. The amount of laser light scattered at approximately 90° angle to the laser beam is called side scatter (SS). A 488 nm dichroic long-pass (DL) filter at a 45° angle to the light path deflects the SS to the SS detector, but transmits fluorescent light of longer wavelengths. Wavelengths <488 nm passing through the 488 DL filter are blocked by a 488 nm laser-blocking filter. The relative magnitude of side scatter is related to the granularity and roughness of the cell surface.

The emission resulting from excitation of fluorescent compounds by laser light can be measured. In general, green, orange, and red fluorophores are used in combination with a 488 nm argon laser to measure various parameters. Fluorescent light with wavelengths >488 nm is transmitted to a series of optical filters which separate the light for three detectors. The first detector (FL1) measures green fluorescent light, which is deflected by a 550 DL filter and passed through a 525 nm band-pass (BP) filter that transmits light between 505 and 545 nm. The second detector (FL2) measures orange fluorescent light, which is deflected by a 600 DL filter and passed through a 575 BP filter that transmits light between 560 and 590 nm. Light with wavelengths >600 nm is transmitted to a 620 BP filter, which transmits light between 605 and 635 nm to the FL3 detector (red fluorescence). With this configuration, it is possible to distinguish and individually measure the magnitude of green, orange, and/or red fluorescence of cells, each correlated with a specific characteristic. More general information on FCM can be found in Shapiro (1994), Ormerod (1994), and Al-Rubeai and Emery (1996).

Fluorescent probe technology

Fluorescence can be defined as the emission of radiation from specific molecules, called fluorophores, after appropriate excitation. A fluorescent probe, also called fluorescent dye, is a fluorophore designed to localize a specific region of a biological specimen or to respond to a specific stimulus (Haugland, 1996).

Fluorescent probes can be divided into two broad categories: those that are used to label other probes, such as antibodies, and those whose fluorescence is related to particular properties of a cell. Fluorescein isothiocyanate (FITC), for instance, is a probe often used for labeling antibodies and has been used in rapid and specific detection methods for microorganisms. A large number of fluorescent probes are available for the study of properties of microorganisms. Frequently used fluorescent probes are shown in Table 1. Basic information on such probes can be found in McFeters et al. (1995), Lloyd and Hayes (1995), and Haugland (1996). Availability of these probes has led to new techniques for assessing viability of microorganisms based on different properties of the cell, such as enzyme activity (Kaneshiro et al., 1993; Diaper and Edwards, 1994; Bunthof et al., 1999); cytoplasmic membrane permeability (Porter et al., 1997; Roth et al., 1997; Williams et al., 1998), membrane potential (Kaprelyants and Kell, 1992; Mason et al., 1995), respiratory activity (Kaprelyants and Kell, 1993); relative DNA content (Sgorbati et al., 1996; Bernander et al., 1998) and pH gradients (Breeuwer et al., 1996; Chitarra et al., 2000).

Assessment of the viability of bacteria

Viability is defined as the capability of a cell to perform all the necessary functions for its survival under given conditions. For viable microorganisms to survive, it is necessary to have an intact cytoplasmic membrane, DNA transcription, RNA translation, enzyme activity, and the capability to reproduce and to grow. Methods for assessment of viability of microorganisms are based on several or all of these requirements.

Plating assays

In plant pathology, several methods are used to test plants and seeds for infection or contamination with plant pathogenic bacteria. Plating assays are the most traditional techniques and are used routinely for detection of viable microorganisms. The conventional plate count method allows isolation of the pathogen by plating plant material, seeds or seed extracts on selective or semi-selective agar media. Subsequently, the isolated bacteria are identified by a range of biochemical or serological tests. However, this method is time-consuming (Lange et al., 1993; Plihon et al., 1995) and skilled technicians are needed to identify each pathogen correctly (Stevens et al., 1997). In addition, the development of colonies on the agar medium may be hampered by cell concentration effects, interference by other microorganisms or presence of inhibitory components in the extract. The use of semi-selective media may reduce the interference of saprophytes on the growth of pathogens, but it can also affect the recovery of the target bacterium (Chun and Alvarez, 1983). Furthermore, bacterial cells which are viable

Table 1. Fluorescent probes[a] commonly used to study properties of bacterial cells by flow cytometry

Characteristic	Probe	Reference
Enzyme activity	FDA	Diaper and Edwards, 1994; Diaper et al., 1992
	cFDA	Diaper and Edwards, 1994; Porter et al., 1997; Nebe von Caron and Baley, 1995
	cFSE	Ueckert et al., 1997; Chitarra et al., 2000
	Calcein AM	Diaper and Edwards, 1994; Kaneshiro et al., 1993
	cF	Bunthof et al., 1999
	ChemChrome B	Diaper and Edwards, 1994; Porter et al., 1997
	BCECF-AM	Diaper and Edwards, 1994; Porter et al., 1997
	$DiOC_6(3)$	Diaper et al., 1992; Mason et al., 1995
Membrane potential	Rh 123	Langsrud and Sundheim, 1996; Kaprelyants and Kell, 1992; Diaper et al., 1992; Porter et al., 1997; Caron and Badley, 1995
	BOX	Caron and Badley, 1995
	$DiBAC_4(3)$	Mason et al., 1995; Williams et al., 1998
Nucleic acid	PI	Bunthof et al., 1999; Kaneshiro et al., 1993; Magarinos et al., 1997; Sgorbati et al., 1996; Ueckert et al., 1997; Caron and Badley, 1995
	DAPI	Sgorbati et al., 1996
	Sytox Green	Langsrud and Sundheim, 1996; Roth et al., 1997
	EB	Caron and Badley, 1995; McClelland and Pinder, 1994a,b
Immunoreagent	FITC	McClelland and Pinder, 1994a,b

[a]Abbreviations: FDA, fluorescein diacetate; cFDA, 5-(and 6-) carboxyfluorescein diacetate; cFSE, 5-(and 6-)-carboxyfluorescein succinimidyl ester; Calcein AM, calcein acetoxy methyl ester; cF, carboxyfluorescein; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; Rh 123, rhodamine 123; BOX, bis-oxonol; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid) trimethine oxonol; PI, propidium iodide; DAPI, 4′,6-diamidino-2-phenylindole; Sytox Green; EB, ethidium bromide; FITC, fluorescein isothiocyanate.

but not culturable are not detected. In spite of the disadvantages of the conventional plate count assays, they are still used in many tests due to their simplicity and effectiveness in providing adequate, but suboptimal, data.

Viability probes

Efficacy of FCM for evaluating the presence of specific plant pathogenic bacteria depends on availability of adequate probes. The probes have to be chosen based on their properties, their fluorescence spectrum and the target microorganism.

Fluorescein diacetate (FDA) is one of the enzyme activity probes that has been used as a cell viability indicator. FDA is a non-fluorescent polar ester compound that can permeate intact cell membranes. Once inside the cell it is hydrolyzed by non-specific esterases to release fluorescein, a polar compound, which is retained inside the cells. The viability can be correlated with the accumulation of fluorescein within the cell (Widholm, 1972). However, fluorescein is poorly retained by viable cells (Edwards et al., 1993), and the FDA method can be hampered by active efflux of fluorescein from the cell. This problem can be minimized by using carboxyfluorescein diacetate (cFDA) or 5 (and 6-)-carboxyfluorescein succinimidyl ester (cFSE) (Breeuwer et al., 1996). The latter fluorescein derivative binds covalently, and is, therefore, retained inside the cells. When observed by fluorescence microscopy, cFSE fluoresces bright green when excited by blue light of 450–490 nm (Figure 2). Propidium iodide (PI) is another probe that has been used to assess viability of microorganisms. PI is a nucleic acid dye that generally does not cross intact cell membranes and, hence, only enters into non-viable cells with damaged membranes. It intercalates into the RNA and DNA backbone independently of base pair ratio (Taylor and Milthorpe, 1980) and A·T-rich regions (Crissman et al., 1979). This is in contrast to DNA dyes such as 4′,6-diamino-2-phenylindole (DAPI) or Hoechst 33342, which bind preferentially to A·T-rich regions (Muller and Gautier, 1975). PI fluoresces red when excited by green light of 515–560 nm (Figure 2).

Rhodamine 123 (Rh 123) is a dye that differentially responds to membrane potential. Most cells maintain a significant electrical potential difference across their membranes at the expense of metabolic energy. Indirect estimates of membrane potential can be obtained by monitoring the difference in concentration of lipophilic cationic indicators or dyes between

cells and the suspending medium. Lipophilic indicators are used, because these molecules pass freely through the lipid portion of the membrane. The concentration gradient of the dye across the membrane is determined by the potential difference across the membrane according to the Nernst equation (Shapiro, 1994). Rh 123 is such a lipophilic cationic membrane potential dye, which enters the cell directly without passing through endocytotic vesicles and lysosomes and is accumulated cytosolically by cells having an internal negative transmembrane electrochemical potential. It has been used to study mitochondria in eukaryotic cells (Skowronek et al., 1990; Rhan et al., 1991) as well as to assess viability of bacteria (Diaper et al., 1992; Davey et al., 1993). The excitation and emission wavelengths of the free and accumulated dye are within the range of 450–560 nm. Since the fluorescence of individual cells stained with Rh 123 can be easily measured by FCM, quantification of uptake by individual cells is possible for large populations of cells (Ronot et al., 1986).

Applications of viability probes in combination with flow cytometry

Flow cytometry has been applied to study bacterial cell cycle kinetics and antibiotic susceptibility (Steen et al., 1982), to enumerate bacteria (Pinder et al., 1990; Page and Burns, 1991), to detect food-borne bacteria (McClelland and Pinder, 1994a,b), to distinguish between viable and non-viable bacteria (Diaper and Edwards, 1994; Mason et al., 1995), to characterize bacterial DNA content (Allman et al., 1992; Christensen et al., 1993), and to characterize fungal spores (Allman, 1992). In the field of plant pathology, this technique is relatively new, and few studies have been performed on the application of FCM for the determination of viability and detection of plant pathogenic bacteria (Chitarra et al., 2000; 2002; Alvarez, 2001).

Diaper and Edwards (1994) studied the colonization of sterile mushroom composts by *Bacillus subtilis* employing FCM in combination with Rh 123 and the enzyme activity probes cFDA and Chemchrome B. FCM was evaluated for detection and enumeration of viable bacteria in filtered compost extract, and also to study the viability of an indigenous compost community. In this study, FCM effectively detected and enumerated *B. subtilis* cells stained with Rh 123, cFDA or Chemchrome B in sterile, filtered compost extract spiked with *B. subtilis*. FCM was not able to detect viable bacteria in indigenous compost populations after staining with cFDA, although it has been shown that this dye is able to stain several species of bacilli. FCM using Rh 123, although underestimating the viable population when compared to the number of colony-forming units, gave similar results as conventional methods with regard to the relative changes in the overall population. FCM using Chemchrome B successfully enumerated the indigenous bacterial population, although higher numbers of viable cells were detected compared to those determined by plate counts.

Porter et al. (1997) also showed that FCM was an effective alternative to microscopy for the detection and viability assessment of bacteria from soil. Viability assessment using fluorogenic ester dyes labeled more viable bacteria than were detected by culture or by 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride reduction. These studies also showed that different soil bacterial populations require different approaches for direct viability assessment. Where the fluorogenic ester dyes are demonstrated to be effective, acceptable enumeration can be achieved using flow sorting. However, a limiting factor and difficult task, in both microscopy and especially FCM, is the separation of bacteria from soil particles without causing cell damage.

Flow cytometry using the fluorescent probe cFSE was applied to assess viability of the plant pathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* by measuring the intracellular pH (pH_{in}). Heat-treated and non-treated *C. michiganensis* subsp. *michiganensis* cells could be distinguished by FCM based on the absence or presence of a pH gradient (Chitarra et al., 2000). Viability of *C. michiganensis* subsp. *michiganensis* cells was also evaluated using the fluorescent probes Calcein AM, cFDA, and PI in combination with FCM (Chitarra, 2001). Heat-treated and viable (non-treated) *C. michiganensis* subsp. *michiganensis* cells labeled with each dye separately or in combination, could be distinguished based on their fluorescence intensity in FCM analysis. Untreated cells showed relatively high green fluorescence levels, whereas damaged cells (heat-treated) showed high red fluorescence levels. FCM allowed rapid quantification and separation of viable *C. michiganensis* subsp. *michiganensis* cells labeled with Calcein AM or cFDA, and heat-treated cells labeled with PI. Although this study was done with pure cultures, it shows the potential of the technique when combined with simultaneous detection of the target cells based on fluorescently labeled antibodies. That will allow determination of

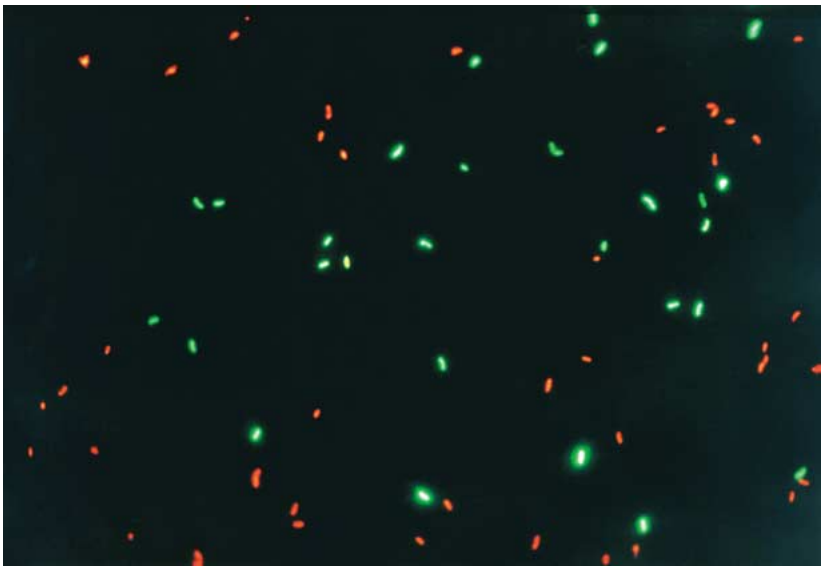

Figure 2. Cells of *C. michiganensis* subsp. *michiganensis* stained by 5 (and 6-)-carboxyfluorescein succinimidyl ester (cFSE) and propidium iodide (PI) and observed by fluorescence microscopy. cFSE stains viable cells, which fluoresce bright green when excited by blue light (450–490 nm). PI particularly stains dead cells, which fluoresce red when excited by green light (515–560 nm).

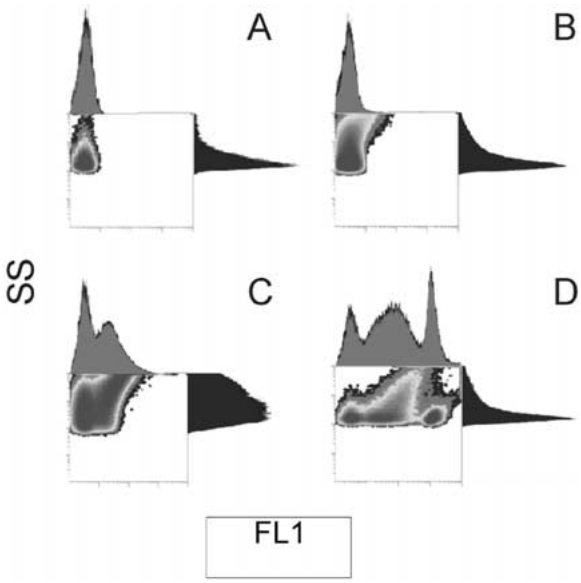

Figure 3. Detection of *X. campestris* pv. *campestris* cells labeled with a mixture of FITC-conjugated monoclonal antibodies (18G12, 2F4, and 20H6) in crude cabbage seed extract by FCM. On the *X*-axis the intensity of the green fluorescence measured by FL1 is indicated and on the *Y*-axis the side scatter (SS). A = phosphate buffer (control); B = seed extract, non-stained; C = healthy seed extract, stained; D = *X. campestris* pv. *campestris* contaminated seed extract, stained.

the viability of specific target cells in plant sap, seed extract, etc.. Indeed, Alvarez (2001) showed that the combination of specific detection of *C. michiganensis* subsp. *michiganensis* with FITC- or PE-labeled monoclonal antibodies and viability determination using the Syber 14 dye was feasible.

Detection of bacteria with flow cytometry

Flow cytometry in combination with fluorescent probe technology has been successfully applied for rapid and specific detection of bacteria in food research, medical and veterinary research, and in environmental studies.

McClelland and Pinder (1994a) used multiparameter FCM as a rapid method for detecting *Salmonella* cells labeled with fluorescent monoclonal antibodies in pure cultures. Accurate detection of specific *Salmonella* serotypes were demonstrated down to levels below 10^4 cells ml^{-1} within 30 min. This level of sensitivity was attained even in the presence of high levels of other bacterial species that could otherwise have interfered with the results. By combining different antibodies, each with a unique fluorescent label, two species could be analyzed simultaneously. They also applied FCM with fluorescently labeled monoclonal antibodies to detect *Salmonella typhimurium* in eggs and milk. By including a sample clearing procedure in their

protocol, a detection limit in the order of 10^3 cells ml^{-1} (total analysis time of 40 min) was achieved. After 6 h of non-selective enrichment, the detection limits were 10 cells ml^{-1} for milk and 1 cell ml^{-1} for eggs, even in the presence of 10,000-fold excess of *Escherichia coli* cells. They concluded that this technique offered advantages of speed and sensitivity for the detection of specific pathogenic bacteria in foods (McClelland and Pinder, 1994b). Similar results were obtained by Kusunoki et al. (1998) for detecting and identifying strains of *E. coli* serotype 0157 : H7 in food ingredients.

Porter et al. (1993) used FCM and cell sorting for the detection and physical separation of *Staphylococcus aureus* and *E. coli* in environmental research. Cells of *S. aureus* were selectively recovered at a purity in excess of 90% when mixed in different proportions with *E. coli*. This was possible even when *S. aureus* composed only 0.4% of the total cells. Cell sorting was tested for the ability to recover *E. coli* from natural lake water and sewage. Populations of *E. coli* released into environmental samples were recovered at purities greater than 90%.

Flow cytometry in combination with FITC-conjugated monoclonal antibodies was evaluated for the detection of *Xanthomonas campestris* pv. *campestris* cells in pure culture, in mixtures of *X. campestris* pv. *campestris* with the common saprophyte *Pseudomonas fluorescens*, and in crude seed extracts (Chitarra et al., 2002). Antibody-labeled *X. campestris* pv. *campestris* cells were rapidly detected at low numbers, i.e. 10^3–10^4 cells ml^{-1}, and the cells could be distinguished from other organisms and contaminating particles based on their high intensity green fluorescence levels (see Figure 3). The density plots represent the number of particles detected by the flow cytometer. The color in these plots gives an indication of the frequency of events. The red areas indicate a high number of particles and the blue areas indicate a low number. In addition, the distribution pattern of the cells detected by the FL1 detector and of the cells detected by the side scatter detector have been indicated on the top and right-hand side of the density plots, respectively. The position of the peaks of the FL1 signal on the *X*-axis is indicative of the intensity of the fluorescence. Unlabeled particles (panels A–C) show low intensity fluorescence levels (background), whereas labeled cells show high fluorescence levels (panel D). By defining regions of interest, exact numbers of cells/particles present in that region can be calculated and target cells can be adequately distinguished from the background.

A prerequisite for the use of antibodies in FCM is the same as for other serological techniques, viz. that no cross-reactions occur with other bacteria present in the sample. The antibodies applied by Chitarra et al. (2002) were previously shown to be specific for *X. campestris* pv. *campestris* and were recommended for use in routine seed health testing (Franken, 1992). Interference with other microorganisms was not observed for the seed lots tested for the presence of *X. campestris* pv. *campestris* with the FCM method. Comparison of FCM with plating on selective media for *X. campestris* pv. *campestris* showed a good correlation for concentrations varying from 10^3 to 10^6 cells ml^{-1}. Overall, the major advantages of the FCM technique in combination with FITC-conjugated antibodies were easy quantification and the short assay time, i.e. less than 1 h.

The advantages and disadvantages of the FCM-based method compared to various other methods available to detect and assess viability of plant pathogenic bacteria are given in Table 2. Compared to other serological techniques used for bacterial testing, the reported sensitivity of about 10^3 cells ml^{-1} is acceptable. This sensitivity was confirmed for detection of *C. michiganensis* subsp. *michiganensis* in seed samples (Alvarez, 2001), and is of the same order of magnitude as the sensitivity reported for immunofluorescence microscopy (IF) (Franken, 1992;

Table 2. Advantages and disadvantages of currently available methods to detect and assess viability of plant pathogenic bacteria

Parameter	Plate count	IF	ELISA	PCR	FCM
Sensitivity	±	+	±	++	+
Specificity	+	±	±	±	±
Cost effectiveness	++	+	+	±	−
Speed	−	±	+	±	++
Labour efficiency	−	±	+	±	+
Skill	±	+	±	+	++
Robustness	+	+	++	±	+

++ = very high; + = high; ± = moderate; − = low.
IF = immunofluorescence microscopy; ELISA = enzyme-linked immunosorbent assay; PCR = polymerase chain reaction; FCM = flow cytometry.
The parameters are based on: Sensitivity: the minimum amount of target detectable; Specificity: degree of false negatives; Cost effectiveness: cost of equipment and lab materials per test; Speed: time required to perform the test; Labor efficiency: amount of personnel input needed to perform a test; Skill: level of competence needed to perform the test; Robustness: tolerance to varying technical and biological conditions.

Van Vuurde and Van der Bovenkamp, 1995), and better than the sensitivity obtained with the enzyme-linked immunosorbent assay (ELISA). In ELISA, a detection limit of 2×10^5 cells ml^{-1} was reported for detecting *X. campestris* pv. *campestris* in infected cabbage leaf tissue with polyclonal antibodies (Alvarez and Lou, 1985). Although the sensitivity of ELISA can be improved, e.g. by using antibodies with better specificity or, in some cases, by heat treatment of the antigen prior to absorption to microtiter plates, a sensitivity of 10^4–10^5 cells ml^{-1} is typical. Leeman et al. (1991), using the immunofluorescence colony-staining method (IFC), reported that the detection level for *Pseudomonas* spp. in undiluted soil sample extracts with a high saprophyte background was between 10 and 100 cells ml^{-1}. However, the method is laborious and specificity of the technique is a concern, because it relies on growth in a selective medium, which can take 1–2 days, colony phenotype, and intensity of staining (Van Vuurde et al., 1995).

Generally, very sensitive detection can be achieved with the polymerase chain reaction (PCR). For example, concentrations of pathogenic bacteria as low as 30 cells ml^{-1} could be confirmed in citrus leaf samples (Miyoshi et al., 1998). However, the PCR technique is only semi-quantitative, is sensitive to interference by saprophytes, and plant samples may contain PCR inhibitors (Schaad et al., 1997).

Concluding remarks

Plant pathologists are faced with the challenge of detecting, enumerating, and assessing viability of plant pathogenic bacteria in plants and seeds. For example, there is a requirement for testing of commercial seed lots for infection or contamination with bacteria. The existing methods for bacterial testing, such as serological techniques the conventional plate assays and, often lead to serious overestimation due to lack of discrimination between live and dead cells, or underestimation due to the presence of viable but non-culturable cells. Conventional assays are slow and necessitate storage of seed lots until results become available. Therefore, a rapid, reliable, and accurate method to detect and to assess viability of plant pathogenic bacteria is highly desirable. The use of fluorescent probes in combination with FCM is a promising new technique. It can be applied to check for the presence of pathogens in plants or in seeds, and to verify what part of the population of the pathogen is viable.

The latest generation of flow cytometers allow simultaneous measurement of three or more fluorescent probes which emit different wavelengths of fluorescence, e.g. green, orange, and red. Thus, simultaneous detection and viability assessment can be performed in the same assay, as was shown by Alvarez (2001) for *C. michiganensis* subsp. *michiganensis*. Testing samples for several pathogens simultaneously is also feasible. Since FCM provides quantitative information about the total number of target cells present in a sample as well as the percentage of viable cells, this information could be valuable for risk assessments and determining the efficacy of seed treatments. With respect to sensitivity, FCM is comparable to current methods such as IF.

Flow cytometers, however, are not simple instruments and basic knowledge of the underlying principles is needed to enable correct interpretation of the results. The quality of the sample, the specific probe and antibodies utilized, the staining procedure, and the settings for the measurements are all important for the precision and accuracy of the measurements. In this respect, there are many similarities with other serological techniques, such as ELISA, IF, or IFC. Because the intensity of the fluorescence signal is measured per particle, FCM is suited to optimize fluorochrome/antibody ratios and to compare the quality of various sources of antibodies. Flow cytometers can be equipped with a flow sorting unit, which can be used for isolation and subsequent characterization of sub-populations of cells in mixed populations. Positively stained cells can be separated from non-stained cells via flow sorting, and can be plated for verification purposes. Furthermore, FCM has the advantage that any combination of analytical parameters can be used to set the criteria for sorting. Disadvantages of the technique, however, are the high cost of the equipment and the need for well-trained technicians. Nonetheless, the higher through put, compared to e.g. plating, and the additional information obtained may make it cost-effective. It is expected that with the growing use of FCM, less expensive models will become available in the near future.

Flow cytometry-based methods have been applied successfully for a diverse range of purposes. In medical research it was used to test vaccine efficacy, to evaluate the interactions between bacteria and phagocytes, to study phagocytic capacity by measuring the uptake of fluorescein-labeled bacteria, and to study effects of intravenous anesthetics on eliminating bacteria from human blood *in vitro* (Wenish et al., 1995; Heller et al., 1998; Rodriguez et al., 2001). In the field of plant

pathology, FCM has been applied in addition to the bacterial studies cited, to measure relative nuclear DNA content of a variety of rust fungal species, including *Puccinia* and *Uromyces* (Eilam et al., 1994), to determine the role of surface components in the infection of plants by pathogenic fungi (Hardham and Suzaki, 1990), and to study spore development and germination (Allman, 1992). FCM may be useful as well for the detection and quantification of fungal spores or to study the effects of chemical treatments on spore populations. FCM should be explored further for applications in the field of plant pathology.

Acknowledgements

We thank Dr. Tjakko Abee, Wageningen University, The Netherlands, for critically reading the manuscript and Ing. Jan Bergervoet, Plant Research International, for providing the FCM figures. We also thank the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES (Brasil) for providing financial support to L.G. Chitarra during this study.

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