Fluorescence techniques to detect and to assess viability of plant pathogenic bacteria

Luiz G. Chitarra

Promotor: prof. dr. ir. F. M. Rombouts
hoogleraar in de levensmiddelenhygiëne en -microbiologie

Co-promotoren: dr. ir. R. W. van den Bulk
Manager business unit Plantontwikkeling en Reproduktie
Plant Research International

dr. T. Abee
universitair hoofddocent bij de leerstoelgroep
Levensmiddelenhygiëne en -microbiologie

Samenstelling promotiecommissie:
prof. dr. ir. P.J.G.M. de Wit (Wageningen Universiteit)
prof. dr. J. da Cruz Machado (UFLA - Brazilië)
dr. A.D.L. Akkermans (Wageningen Universiteit)
dr. ir. J.M. van der Wolf (Plant Research International)

Fluorescence techniques to detect and to assess viability of plant pathogenic bacteria

Luiz G. Chitarra

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. Ir. L. Speelman,
in het openbaar te verdedigen
op 28 maart 2001
des namiddags te half twee in de Aula

This work was carried out at Plant Research International, Business Unit Plant Development and Reproduction, P.O. Box 16, 6700 AA, Wageningen, The Netherlands, and at Wageningen University, Department of Agrotechnology and Food Sciences, P.O. Box 8129, 6700 EV, Wageningen, The Netherlands.
The research described in this thesis was financially supported by CAPES (Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil).

L. G. Chitarra - Fluorescence techniques to detect and to assess viability of plant pathogenic bacteria - 2001
Ph.D Thesis Wageningen University, Wageningen, The Netherlands - With summaries in English and Dutch.
Key words: Plant pathogenic bacteria / *Clavibacter michiganensis* subsp. *michiganensis* / *Xanthomonas campestris* pv. *campestris* / fluorescence techniques / detection methods / viability

ISBN: 90 - 5808 - 389 - 6
Printed by: Ponsen & Looijen BV, Wageningen

Propositions

1. Plant pathologists should use methods for rapid detection and assessment of the viability of plant pathogens in one and the same assay.
   This Thesis.

2. It is difficult to define and to determine the viability of non-culturable bacteria.
   This Thesis.
   Van Vuurde, W. L., Kastelein, P. and Van der Wolf, J. M. (1995) Immunofluorescence colony-staining (IFC) as a concept for bacterial detection in quality testing of plant materials and ecological research. *EPPO Bulletin* **25:** 157-162.
   Barer, M., Bogosian, G., Kell, D. and Williams, H. (2000) Resuscitating a logical approach to viability in the face of an "Eastern Wind". ASM *News* **66 (7):** 381-382.

3. Understanding the viability of bacterial pathogens facilitates designing relevant risk assessment models.
   This Thesis.

4. The Calcein AM or PI double-staining method is reliable for viability assessment of a variety of protozoans, however, this method does not provide the same viability information on yeasts and bacteria.
   Kaneshiro, E. S., Wyder, M. A., Wu, Y. P. and Cushion, M. T. (1993) Reliability of calcein acetoxy methyl ester and ethidium homodimer or propidium iodide for viability assessment of microbes. *Journal of Microbiological Methods* **17:** 1-16.

5. The tendency in flow cytometry towards multi-colour analysis ensures the companies producing instruments and fluorescent probes that there is a pot of gold at the end of the rainbow.

6. Indistinctness about colour compensation in flow cytometry starts with the spelling of the word.

7. Brazilians are able to adapt to different cultures, but they never lose their identity.

8. The lack of sunshine in The Netherlands influences the behavior of the Dutch people.

Propositions belonging to the Doctoral Thesis "Fluorescence techniques to detect and to assess viability of plant pathogenic bacteria" by Luiz G. Chitarra, Wageningen, March 28, 2001.

Preface

I would like to express my sincere gratitude to those who contributed to accomplishing all the work that has been presented in this thesis.

I am deeply grateful to my Promotor, Prof. Dr. Frans M. Rombouts, from the Department of Food Technology and Nutritional Sciences at the Wageningen University, The Netherlands, for his wise advices and support.

My sincere thanks go to my Co-promotors Dr. Ruud van den Bulk from Plant Research International and Dr. Tjakko Abee from the Wageningen University, The Netherlands, for their supervision, assistance, attention, suggestions and criticism to my daily research activities and the manuscripts which form the basis of this thesis.

I am also thankful to Pieter Breeuwer and Jan Bergervoet for their technical assistance in the laboratories and valuable suggestions to my research project.

My very special thanks goes to Dr. Kees Langerak, for his support, wise advices, suggestions and our friendly relationship through all these years.

My acknowledgements should go to the " Ladies of the Phytopathology Lab " at Plant Research International, Patricia van der Zouwen, Carin van Tongeren, and Yvonne Birnbaum for their friendship and technical assistance. I also would like to thank Rob van der Schoor and Henk Jalink for their computer assistance, and Patrick Verbaarschot from the Food Microbiology Lab, Wageningen University, for his technical assistance.

I wish to express my sincere thanks to all other colleagues at Plant Research International and at the Department of Food Technology and Nutritional Sciences at Wageningen University.

I extend my gratitude to the Federal University of Lavras (UFLA), Brazil, in particular to Prof. Dr. José da Cruz Machado and Prof. Dr. Fabiano Ribeiro do Valle for stimulating me to go to Wageningen for my Ph.D.

I am deeply grateful to the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES - Brazil) for their financial support during this study.

I would like to express my sincere appreciation to the Brazilian and Portuguese communities in Wageningen for their support and friendship. I extend my

gratitude to some special Dutch friends: Lenny, Noranne, and Nelleke Langerak, Peter, Twan and Alice.

I am greatly thankful to my mother, Maria da Gloria Chitarra, and my brothers, especially Admilson Bosco Chitarra and my sister-in-law Maria Isabel Fernandes Chitarra, who always encouraged, motivated and supported me in my research work.

Finally, I wish to express my very special thanks, my love and gratitude to my wife Gilma Silva Chitarra, my daughter Cristiane Silva Chitarra and my son Guilherme Silva Chitarra, who fundamentally contributed to the achievement of this work.

I dedicate this thesis to

Gilma, Cristiane and Guilherme

Luiz

Contents

Outline of this thesis

The conventional methods to detect and to assess the viability of plant pathogenic bacteria are usually based on plating assays or on serological techniques. Plating assays provide information about the number of viable cells, however, are time-consuming and laborious. Serological methods such as 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, are dependent on the specificity of the antibodies, and are not sensitive enough. Flow cytometry (FCM) in combination with fluorescent probes technology is in principle a rapid, sensitive, quantitative and therefore, a promising technique to detect and to assess the viability of microorganisms. FCM has been applied successfully to detect and distinguish between viable and non-viable bacteria in the fields of food microbiology, veterinary science and medical research. The aim of this thesis was to explore the potential of FCM in the field of plant pathology. The work described in this thesis focuses on the development of FCM technique in combination with fluorescent probes and specific antibodies for a rapid, reliable, and accurate detection and assessment of the viability of plant pathogenic bacteria.

Chapter 1 is a literature overview of a range of applications of flow cytometry as a new tool for a rapid detection and assessment of the viability of microorganisms.

In **Chapter 2** the viability of *Clavibacter michiganensis* subsp. *michiganensis* cells was determined by measuring the intracellular pH as a parameter for viability, applying the fluorescent probe 5(and 6-)-carboxyfluorescein succinimidyl ester (cFSE) in combination with fluorescence spectrofluorometry and flow cytometry.

In **Chapter 3** the viability of *Clavibacter michiganensis* subsp. *michiganensis* cells was assessed applying the enzyme activity probes carboxyfluorescein diacetate (cFDA), Calcein acetoxy methyl ester (Calcein AM), and the nucleic acid probe Propidium iodide (PI) in combination with flow cytometry.

In **Chapter 4** the flow cytometry technique was evaluated for a rapid detection of *Xanthomonas campestris* pv. *campestris* cells with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies in pure cultures and in suspensions containing both Xcc and the common saprophyte *Pseudomonas fluorescens* (Psf).

Chapter 5 describes the detection of *Xanthomonas campestris* pv. *campestris* in crude extracts of naturally contaminated seed lots applying FITC-labeled monoclonal antibodies and flow cytometry. Results were compared with those of the routinely used plating method and immunofluorescence microscopy.

In **Chapter 6** the findings of this research and the perspectives of applying FCM in combination with fluorescent probes in the field of plant pathology are discussed.

Chapter 1

New and current techniques to detect and to assess viability of plant pathogenic bacteria.

L.G.Chitarra[1,2] and R.W. van den Bulk[1]

[1]Plant Research International B.V., P.O. Box 16, 6700 AA, Wageningen, The Netherlands, and [2]Department of Food Technology and Nutritional Sciences, Wageningen University and Research Center, Wageningen, The Netherlands

1 Introduction

Plant bacteriology became a science in the second half of the 19th century. In 1878, Burrill in Illinois, USA, showed that fire blight disease of pear and apple was caused by a bacterium. Shortly afterwards, Erwin F. Smith from the U.S Department of Agriculture (USDA), showed that several other plant diseases were also caused by bacteria (Agrios 1997). Advances in the field of plant pathogenic bacteria in particular took place during the last four decades.

Nowadays it is known that about 100 species of bacteria are able to cause diseases in plants (Agrios 1997). Plant pathogenic bacteria may develop in the host plant as parasites, on the plant surface as epiphytes, or in plant debris or in the soil as saprophytes.

The dissemination of plant pathogenic bacteria primarily occurs by water, insects, animals, humans, and seeds. In cases in which bacteria infect or infest the seeds of their host plants, they can be carried for short or long distance by any means of seed dispersal. When seeds are infected or infested with bacteria, they can cause major economic losses in crop production. To avoid this, seeds and plants must be free of pathogens.

Microbial testing for the presence of harmful bacteria is commonly done by isolation and culturing on artificial growth media followed by identification. Depending on the organism, this is a very time-consuming process, taking from 2 days up to one or more weeks. In spite of using semi-selective culture media, other

microorganisms and product components may interfere by overgrowing or suppressing outgrowth of the target microorganism.

Current methods that are also routinely used for bacterial detection include immunological and DNA techniques. Both techniques can be performed in a shorter timespan than most plating assays. However, these techniques are considered to provide the user only with semi-quantitative information, which for various tests is not satisfactory. Moreover, both techniques do not distinguish between viable and dead cells of the target organism, whereas such information is essential for an effective decision-making, e.g. on allowing import and export or on treatments of seed lots. Both quantitative data and information about viability provide the basis for risk assessment of disease development.

This review will focus on new techniques for detection and for the assessment of viability of plant pathogenic bacteria, based on flow cytometry (FCM) and fluorescent probe technology, and the existing methods, such as the plate count technique, immunofluorescence (IF), enzyme-linked imunosorbent assay (ELISA), immunofluorescence colony-staining (IFC), and the polymerase chain reaction (PCR).

2 Flow Cytometry

Flow cytometry is a process in which physical and / or chemical characteristics of biological particles are measured while passing through a measuring apparatus in a fluid stream (Shapiro 1988). It is a technique that has the ability to measure several parameters on thousands of individual cells within a few minutes (Muirhead *et al.* 1985). The parameters measured by a flow cytometer are fluorescence and light scattering. The filter configuration of a typical flow cytometer is shown in Fig. 1. This configuration is for instance used in an Epics XL - MCL (Coulter Corporation, Miami, Florida - USA). As the cells pass through the sensing area of the flow cell, the laser beam illuminates the fluorescent dyes attached to or contained in the cells. Various parameters can be measured. Forward scatter (FS) is the amount of laser light scattered at narrow angles to the axis of the laser beam, and it is proportional to the size of the cell. The amount of laser light scattered at approximately 90° angle to the axis of the laser beam is called side scatter (SS), which is related to the granularity of the cell that scattered the laser light. In order for the sensors to measure SS and the Fluorescent Light of the dyes used (FL), the light must be collected and the SS and FL

must be separated. SS is separated first from the output of the pickup lens/spatial filter assembly using a 488 nm dichroic long-pass (DL) filter at a 45° angle to the light path. The 488 DL filter reflects the SS to the SS sensor, but transmits fluorescent light of longer wavelenghts. The light that the 488 DL filter transmits goes to a 488 nm laser-blocking (BK) filter. The 488 BK filter blocks any remaining laser light, transmitting only the fluorescent light, and the optical filters separate the light for the three sensors. A 550 DL filter reflects light less than 550 nm to a 525 nm band-pass (BP) filter that transmits light between 505 nm and 545 nm to the FL1 Sensor (green fluorescence). A 600 DL filter reflects light between 555 nm and 600 nm to a 575 BP filter, which transmits light between 560 nm to 590 nm to the FL2 sensor (orange fluorescence). The light between 605 nm and 725 nm is transmited to a 620 BP filter, which transmits light between 605 nm and 635 nm to the FL3 sensor (red fluorescence). With this configuration it is possible to distinguish and measure green, orange, and red fluorescent probes.

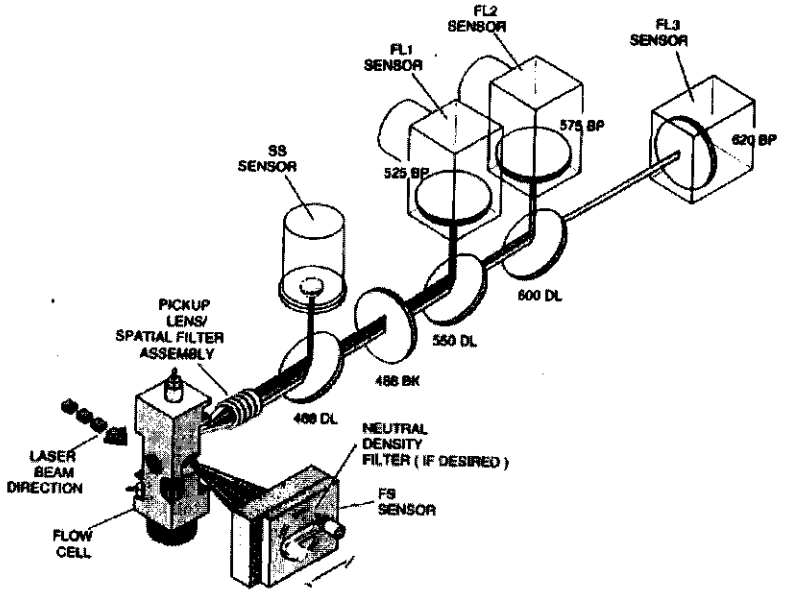

Fig. 1. Schematic representation of an Epics XL - MCL (Coulter Corporation, Miami, Florida - USA) flow cytometer showing filter configuration and fluorescence sensors (FL1, FL2, and FL3). The green, orange, and red fluorescence are measured by FL1, FL2, and FL3 sensors respectively.

3 Fluorescent Probe Technology

Fluorescence can be defined as the emission of radiation that occurs in certain molecules called fluorophores or fluorescent dyes. A fluorescent probe is a fluorophore designed to localize a specific region of a biological specimen or to respond to a specific stimulus (Haugland 1996).

Table 1. Fluorescent probes[a] commonly used to detect and to assess viability by flow cytometry.

Characteristics	Probes	Reference
Enzyme activity	FDA	Diaper and Edwards 1994; Diaper, Tither, and Edwards 1992
	cFDA	Diaper and Edwards 1994; Porter *et al.* 1997; Caron and Badley 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, Tither, and Edwards 1992; Mason *et al.* 1995
Membrane potential	Rh 123	Langsrud and Sundheim 1996; Kaprelyants and Kell 1992; Diaper, Tither, and Edwards 1992; Magarinos *et al.* 1997; Porter *et al.* 1997; Caron and Badley 1995
	BOX	Caron and Badley 1995
	$DiBAC_4(3)$	Mason *et al.* 1995; Willians *et al.* 1999
Nucleic acid	PI	Bunthof *et al.* 1999; Kaneshiro *et al.* 1993; Magarinos *et al.* 1997; Sgorbati *et al.*1996; Miller and Quarles 1990; Ueckert *et al.* 1997; Caron and Badley 1995
	DAPI	Sgorbati *et al.* 1996
	Sytox Green	S. Langsrud and G. Sundheim 1996; Roth *et al.* 1997
	EB	Caron and Badley 1995; McClelland and Pinder 1994a, 1994b
Immunoreagent	FITC	McClelland and Pinder 1994a, 1994b; Chapters 4, 5

[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.

The fluorescent probes can be divided into two broad categories, those that are used to label other probes (often antibodies) covalently and those whose fluorescence is related to particular properties of the cells. Over the past two decades, the development of these fluorescent probes has led to new techniques to detect and to assess the viability of microorganisms. Fluorescein isothiocyanate (FITC) is a probe often used for labeling antibodies and has been used for a rapid and specific detection of microorganisms. Fluorescent probes have also been developed to explore different properties of the cell, such as enzyme activity (Kaneshiro *et al.* 1993; Diaper and Edwards 1994; Endo *et al.* 1997; Ueckert *et al.* 1997; Bunthof *et al.* 1999); cytoplasmic membrane permeability (Magariños *et al.* 1997; Porter *et al.* 1997; Roth *et al.* 1997; Williams *et al.* 1998), membrane potential (Kaprelyants and Kell 1992; Mason *et al.* 1995; Langsrud and Sundheim 1996), respiratory activity (Kaprelyants and Kell 1993a, 1993b); relative DNA content (Allman *et al.* 1992; Christensen *et al.* 1993; Sgorbati *et al.* 1996; Bernander *et al.* 1998) and pH gradients (Breeuwer *et al.* 1996; Chitarra *et al.* 2000). A large number of fluorescent probes are available for labeling microorganisms, and the most used fluorescent probes to detect and to assess viability of bacteria, protozoa, mammalian cells, yeast and some fungi 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).

4 Assessment of the viability of bacteria

Viability can be 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 these requirements.

4.1 Plating assays

In plant pathology, several methods are used to test plants and seeds for contamination with plant pathogenic bacteria. Plating assays are the most traditional techniques, and are used routinely for detection and assessment of the viability of 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 (Schaad and Donaldson 1980; Randhawa and Schaad 1984; Chang *et al.* 1990; Chang *et al.* 1991; Shirakawa *et al.* 1991; De la Cruz *et al.* 1992; Goszczynska and Serfontein 1998). Subsequently, the isolated organisms 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 results are sometimes difficult to interpret due to cell concentration effects, interference by other microorganisms or presence of inhibitory components in the extract. Semi-selective media may reduce the interference of saprophytes, but it can also affect the recovery of the target bacterium (Chun and Alvarez 1983). Furthermore, cells which are dormant, non-culturable or sub-lethally damaged 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.

4.2 Viability probes

Today, promising tools to assess microbial viability are fluorescent probes. The probes have to be chosen based on their properties, fluorescence spectrum, and 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 cleaved (hydrolysed) by non-specific esterases to release fluorescein, a polar compound, which is retained inside the cells. Thus, the viability can be correlated with the ability of the cell to accumulate fluorescein (Widholm 1972; Gahan 1984). However, fluorescein is poorly retained by viable cells (Fry 1990; Edwards *et al.* 1993), and the FDA method can be easily frustated due to an active efflux of fluorescein to the extracellular environment. This problem can be minimized by using, for instance, carboxyfluorescein diacetate (cFDA) (Chapter 3) or 5 (and 6-)-carboxyfluorescein succinimidyl ester (cFSE) (Breeuwer *et al.* 1996). The latter, fluorescein derivative binds covalently, and is, therefore, better retained inside the cells. When observed in fluorescence microscopy, cFSE fluoresces bright green when excited by blue light of 450 - 490 nm.

Propidium iodide (PI) is a nucleic acid probe that has been used to assess viability of microorganisms. PI is a dye that is not supposed to cross intact cell

membranes (Alvarado-Aleman *et al.* 1996). It passes through damaged cell membranes and intercalates into the RNA and DNA backbone independently of base pair ratio (Hudson *et al.* 1969; 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.

Rhodamine 123 (Rh 123) is a membrane potential dye that has been used to assess viability of microorganisms. 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 distribution of lipophilic cationic indicators or dyes between cells and the suspending medium. Lipophilic indicators are used, because this characteristic enables indicator molecules to pass freely through the lipid portion of the membrane; thus the concentration gradient of an indicator across the membrane is determinated by the potential difference across the membrane according to the Nernst equation (Shapiro 1988). Rhodamine 123 is such a lipophilic cationic membrane potential dye, which enters the cell directly without passing through endocytotic vesicles and lysosomes (accumulated cytosolically by cells showing inside a 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 dye and the dye taken up by the cells are within a range of 450 to 560 nm. Since the fluorescence of individual cell stained with Rh 123 can be easily measured by flow cytometry, quantification of the uptake of the dye per cell is possible for large populations of cells (Ronot *et al.* 1986).

4.3 Applications of viability probes in combination with flow cytometry

In the field of microbiology, flow cytometry (FCM) 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, 1994b), 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; Chapters 3, 4). The disadvantages of this technique, however, are the high cost of the equipment and the fact that well-trained technicians are needed to set up the protocols, before it can be routinely applied.

Diaper and Edwards (1994) studied the colonisation of sterile mushroom composts by *Bacillus subtilis* employing flow cytometry in combination with the membrane potential dye rhodamine 123 (Rh 123), and the enzyme activity probes, cFDA and Chemchrome B. FCM was evaluated with respect to detect and enumerate viable bacteria in filtered compost extract, and also to study the viability of an indigenous compost community. The results showed that FCM was able to detect and enumerate *Bacillus subtilis* cells stained with Rh 123, cFDA or Chemchrome B in sterile, filtered compost extract spiked with *Bacillus subtilis*. In indigenous compost populations, FCM was not able to detect any viable bacteria after staining with cFDA, although it has been shown that cFDA is able to stain several species of bacilli. Rh 123, although underestimating the viable population when compared to CFU's determined by plating, gave a similar correlation with regard to the relative changes in the overall population. Chemchrome B was considered successful to enumerate the indigenous bacterial population, although FCM detected higher numbers of viable cells compared with those determined by plate counts.

In the field of plant pathology, FCM combined with fluorescent probes technique was applied to assess viability of the plant pathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) by measuring the intracellular pH (pH_{in}) as a viability parameter. The pH_{in} of Cmm was determined using FCM and the fluorescent probe 5(and 6-)-carboxyfluorescein succinimidyl ester (cFSE). Heat-treated and non-treated Cmm cells could be distinguished by FCM based on the absence and presence of a pH gradient, respectively (Chitarra *et al.* 2000). The assessment of viability of Cmm cells was also evaluated using the fluorescent probes Calcein AM, cFDA, and PI in combination with flow cytometry (Chapter 3). Heat-treated and viable (non-treated) Cmm cells labeled with Calcein AM, cFDA, PI, or combinations of Calcein AM and cFDA with PI, could be

distinguished based on their fluorescence intensity in flow cytometry analysis. Non-treated cells showed relatively high green fluorescence levels, whereas damaged cells (heat-treated) showed high red fluorescence levels. Flow cytometry allowed a rapid quantification and separation of viable Cmm cells labeled with Calcein AM or cFDA, and heat-treated cells labeled with PI.

5 Current techniques for detecting plant pathogenic bacteria

Apart from plating assays mentioned earlier, current methods for detection of plant pathogenic bacteria are based on serological techniques, such as enzyme-linked immunosorbent assay (ELISA), immunufluorescence microscopy (IF) or Immunofluorescence colony-staining (IFC), or on DNA techniques, such as the polymerase chain reaction (PCR). Their advantages and disadvantages will briefly be discribed.

5.1 Serological techniques

5.1.1 ELISA

The enzyme-linked immunosorbent assay (ELISA) registers the occurrence of antigen-antibody complexes by a rapid enzymatic development of a distinctly coloured product. It was potentially recognized as useful for the detection of plant pathogenic bacteria after the report written by Engvall and Perlmann in 1971. Many variations of this procedure have been developed and the most important ones nowadays are the direct assay using the double antibody sandwich method (DAS-ELISA), and the indirect assay, where a secondary antibody, usually an antiglobulin conjugated with enzyme is added after addition of the primary antibody. In some cases, indirect ELISA is preferred rather than DAS-ELISA, since the secondary antibody-enzyme conjugate can be obtained commercially and it saves the time needed to produce and to conjugate the antibody and its enzyme. It is also preferred in situations where antibodies do not react efficiently as trapping antibodies. The advantages of the ELISA method for the detection of plant pathogenic bacteria are its speed, i.e. 4-5 hours required to perform the assay comparing to other methods of detection, its simplicity, and it is especially useful for routine diagnosis in a large number of samples. However, ELISA lacks sensitivity. A detection limit of 2×10^5 cells ml^{-1} testing infected cabbage leaf tissue was reported for ELISA using

polyclonal antibodies against *Xanthomonas campestris* pv. *campestris* (Alvarez and Lou 1985). The sensitivity of the ELISA test applied to detect *Clavibacter michiganensis* subsp. *sepedonicum* in potato tubers using monoclonal antibody was determined by testing serial 10-fold dilutions of preparations made from tubers with typical ring rot symptoms. Sample dilutions up to 10^6 times were still positive in ELISA (De Boer *et al.* 1988). The detection limits of ELISA can be improved in a stronger ELISA reaction by using antibodies with better specificity, and in some cases, when the antigen is heat-treated prior to absorption to microtiter plate wells (Alvarez and Lou 1985; Kishinevsky and Gurfel 1980).

5.1.2 Immunofluorescence microscopy (IF)

Immunofluorescence microscopy is a method used to detect or to confirm plant pathogenic bacteria in seeds and plants. The antibody is bound to a fluorescent probe, usually fluorescein isothiocyanate (FITC), and stained bacterial cells can be visualized on microscope slides with an appropriately equipped fluorescence microscope. The IF method has been used to detect bacteria in seeds (Franken 1992; Van Vuurde and Bovenkamp 1995) and the sensitivity of the method has been reported to be up to 10^3 cells ml^{-1} (Taylor 1978). The advantage of the IF method is that it offers the possibility to study cell morphology in combination with serological reaction. However, the reliability of this method, as well as ELISA, depends on the specificity of the antibodies, which must be tested prior to use and preferably should not cross-react with other bacteria present in the sample. In general, the disadvantages of this technique are: 1) it does not discriminate between live and dead cells; 2) it depends on the quality of the antiserum; 3) the examination of IF slides is time consuming and should be performed by experienced technicians.

5.1.3 Immunofluorescence colony-staining (IFC)

Immunofluorescence colony-staining is based on a combination of plating and serological techniques. It is a technique that allows the colonies of the target bacterium to be distinguished from those of other microorganisms after growth in an agar-medium, followed by drying the medium and adding specific FITC-conjugated antibodies to label the target bacterium. The target colonies fluoresce against a dark background when observed with a fluorescence microscope. This technique provides a tool for the study of plant pathogenic bacteria, such as the *in situ* detection of target

bacteria on or in plant tissues, i.e. roots, stems or seeds. It is used to distinguish between culturable and non-culturable cells, because observations are done on (micro) colonies. It is possible to detect different target bacteria through the application of different fluorescent markers. Furthermore, IFC is also used for selective isolation of cross-reacting bacteria for research purposes, thereby improving the reliability of serological techniques (Van Vuurde *et al.* 1995). Leeman *et al.* (1991), using the IFC method, 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}. The detection level for soft rot *Erwinia* ssp. using undiluted tissue culture extract was determined to be about 10^2 CFU ml^{-1} (Van Vuurde and Roozen 1990). It seems that a detection level of approximately 10^2 CFU ml^{-1} can be achieved with IFC. However, a major concern is the specificity of the technique. The specificity of IFC relies on growth in a selective medium, which can take 1 to 2 days, colony phenotype, and serological staining characteristics (Van Vuurde, Kastelein and Van der Woff 1995).

In general, serological techniques play an important role in the identification and detection of plant pathogenic bacteria. The main advantage of serological tests are their low costs. Disadvantages of these techniques are the risk of cross-reaction, the fact that they do not distinguish between viable and non-viable cells (except for IFC), and the tests being time consuming and laborious.

5.2 Polymerase chain reaction (PCR)

The polymerase chain reaction is a technique which involves the amplification of a specific piece of DNA from the genome of a target bacterium. PCR-based methods may be rapid, highly sensitive, accurate, and specific for detection and identification of plant pathogenic bacteria (Rasmussen and Wulf 1991; Prosen *et al.* 1993; Firrao and Locci 1994; Rademaker and Janse 1994; Dreir *et al.* 1995; Audy *et al.* 1996; Cajza *et al.* 1996; Lopes and Damann 1997; Santos *et al.* 1997; Fegan *et al.* 1998; Manulis *et al.* 1998; Pan *et al.* 1998; Toth *et al.* 1998; Verdier *et al.* 1998; Wang *et al.* 1998; Belgrader *et al.* 1999; Cubero *et al.* 1999; Oh *et al.* 1999; Toth *et al.* 1999) as well as for detecting culturable target cells when applied in combination with enrichment as in bio-PCR (Schaad *et al.* 1995; Manulis *et al.* 1999; Wang *et al.* 1999). Using PCR, the presence of pathogenic microorganisms in very low concentrations in samples, i.e. 30 CFU ml^{-1}, could be confirmed (Miyoshi *et al.* 1998). The

disadvantage, however, as is the case for serological assays, DNA methods do not, or not completely, provide information about the viability of the target pathogens. Furthermore, when natural plant samples are used this technique may lack sensitivity due to the presence of PCR inhibitors, potentially resulting in false negatives (Schaad *et al.* 1997). PCR technique is semi-quantitative and relatively sensitive to interference of saprophytes (Van Vuurde 1997).

6 Detection of bacteria applying flow cytometry

Flow cytometry in combination with fluorescent probes technology has been successfully applied to assess the viability of microorganisms as well as for a rapid and specific detection of bacteria in medical and veterinary research, and in environmental samples (Diaper *et al.* 1992; Li and Walker 1992; May *et al.* 1994; Porter *et al.* 1993; McClelland and Pinder 1994a, 1994b; Pinder and McClelland 1994; Porter *et al.* 1997; Kusunoki *et al.* 1998; Bunthof *et al.* 1999).

McClelland and Pinder (1994a) used multiparameter flow cytometry as a rapid method for detection of Salmonella cells labeled with fluorescent monoclonal antibodies in pure cultures. Accurate detection of specific *Salmonella* serotypes was demonstrated down to levels below 10^4 cells ml^{-1} within 30 minutes. 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. With combinations of different antibodies, each with a unique fluorescent label, simultaneous analysis for two species was possible. They also applied flow cytometry with fluorescently labeled monoclonal antibodies to detect *Salmonella typhimuruim* in eggs and milk, and concluded that this technique offered advantages of speed and sensitivity for the detection of specific pathogenic bacteria in foods (McClelland and Pinder 1994b).

The first report on the detection of a plant pathogenic bacterium applying FCM in combination with antibodies is described in this thesis (Chapters 4). FCM was evaluated for the detection of *Xanthomonas campestris* pv. *campestris* (Xcc) cells labeled with FITC-conjugated monoclonal antibodies. This was done in pure culture, in mixed cultures with the common saprophyte *Pseudomonas fluorescens* (Psf), and in crude seed extracts. Antibody-labeled Xcc cells could rapidly be detected at low numbers, i.e. 10^3 cells ml^{-1}, and the cells could also be distinguished from other organisms or particles present in the samples based on their high intensity green

fluorescence levels. The only prerequisite for the use of antibodies is the same as for the other serological techniques discussed, viz. that no cross-reactions with other bacteria present in the sample occur. The antibodies applied in this study (Chapter 5) were previously shown to be specific for Xcc and are recommended for use in routine seed health testing for Xcc (Franken 1992). One of the major advantages of applying the FCM technique in combination with FITC-conjugated antibodies for the detection of Xcc is the short assay time, i.e. less than 1 hour, and the easy quantification.

7 Concluding remarks

Plant pathologists are among other things faced with the challenge of detecting, enumerating, and assessing viability of plant pathogenic microorganisms in plants and seeds. The existing methods, such as the conventional plate assays and serological techniques, often lead to serious overestimation, due to not discriminating between live and dead cells, or underestimation, due to the presence of viable but non-culturable cells, of the bacterial population present in the sample. These methods are neither suitable to test disinfected seeds nor for a rapid detection of plant pathogens.

The importance and the need for methods to detect and to assess viability of plant pathogenic microorganisms accurately, reliably and fast was discussed. A promising new technique is the use of fluorescent probes in combination with FCM (Chitarra *et al.* 2000; Chapters 3, 4, 5). It can be applied to check for the presence of pathogens in plants or plant parts, and to verify if the population of the pathogen is viable, non-viable, or maybe dormant.

The latest flow cytometers allow measuring of two or three different fluorescent probes based on their emission of wavelengths meaning that simultaneous detection and viability assessment can be performed in the same assay. FCM also provides quantitative information about the total number of target cells present in a sample as well as the percentage of viable cells.

A flow cytometry-based method has technical benefits over traditional methods, since it is rapid, relatively sensitive, and quantitative for the detection and determination of viability of microorganisms and should be further explored for applications in the field of plant pathology.

References

Agrios, G.N. (1997) Plant Pathology. 4th ed. New York: Academic Press. p. 635.

Allman, R., Hann, A.C., Manchee, R. and Lloyd, D. (1992) Characterization of bacteria by multiparameter flow cytometry. *Journal of Applied Bacteriology* **73:** 438-444.

Allman, R. (1992) Characterisation of fungal spores using flow cytometry. *Mycol. Res.* **96:** 1016-1018.

Alvarado-Aleman, F.J., Collazo-Ponce and Kumate-Rodrigues, J. (1996) Assessment of viability of yeast by flow cytometry using light green as vital dye exclusion. *Rev. Lat.-Amer. Microbiol.* **38:** 25-30.

Alvarez, A.M. and Lou, K. (1985) Rapid identification of *Xanthomonas campestris* pv. *campestris* by ELISA. *Plant Disease* **69:** 1082-1086.

Audy, P., Braat, C.E., Saindon, G., Huang, H.C. and Laroche, A. (1996) A rapid and sensitive PCR-based assay for concurrent detection of bacteria causing common and halo blights in bean seed. *Phytopathology* **86:** 361-366.

Belgrader, P., Benett, W., Hadley, D., Richards, J., Stratton, P., Mariella, R.Jr. and Milanovich, F. (1999) PCR detection of bacteria in seven minutes. *Science* **284 (5413):** 449-450.

Bernander, R., Stokke, T. and Boye, E. (1998) Flow cytometry of bacterial cells: comparison between different flow cytometers and different DNA stains. *Cytometry* **31:** 29-36.

Breeuwer, P., Drocourt, J.L., Rombouts, F.M. and Abee, T. (1996) A novel method for continuous determination of the intracellular pH in bacteria with the internally conjugated fluorescent probe 5 (and 6-)-carboxyfluorescein succinimidyl ester. *Applied and Environmental Microbiology* **62:** 178-183.

Bunthof, C.J.,Van den Braak, S., Breeuwer, P., Rombouts, F.M. and Abee, T. (1999) Rapid fluorescence assessment of the viability of stressed *Lactococcus latis*. *Applied and Environmental Microbiology* **65:** 3681-3689.

Burrill, T.J. (1878) *Trans. Ill. State Hortic. Soc.* **11:** 79-80.

Cajza, M., Rezler, A. and Pospieszny, H. (1996) Identification of *Clavibacter michiganensis* subsp. *michiganensis* by the polymerase chain reaction (PCR). *Journal of Plant Protection Research* **37:** 45-51.

Chang, C.J., Donaldson, R., Crowley, R. and Pinnow, D. (1990) Isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds using a highly selective medium. In: Klement, Z. (ed.). *Proceedings of the* 7th *International Conference on Plant Pathogenic Bacteria*. Hungary, Budapest: Akadémiai Kiadó. pp. 533-539.

Chang, C.J., Donaldson, D., Crowley, M. and Pinnow, D. (1991) A new semiselective medium for the isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds. *Phytopathology* **81:** 449-453.

Chen, L.C., Chen, T.Z., Chen, H.L. and Yeh, Y. (1998) Establishment of molecular markers for the detection and diagnosis of *Botrytis cinerea and B. elliptica. Plant Pathology Bulletin* **7(4):** 177-188.

Chitarra, L.G., Breeuwer, P., Van den Bulk, R.W. and Abee, T. (2000) Rapid fluorescence assessment of intracellular pH as a viability indicator of *Clavibacter michiganensis* subsp. *michiganensis*. *Journal of Applied Microbiology* **88:** 809-816.

Christensen, H., Bakken, L.R. and Olsen, R.A. (1993) Soil bacterial DNA and biovolume profiles measured by flow-cytometry. *FEMS Microbiology Ecology* **102:** 129-140.

Chun, W.W.C. and Alvarez, A.M. (1983) A starch-methionine medium for isolation of *Xanthomonas campestris* pv. *campestris* from plant debris in soil. *Plant Disease* **67:** 632-635.

Crissman, H.A., Orlicky, D.J. and Kissane, R.J. (1979) Fluorescent DNA probes for flow cytometry. Considerations and prospects. *J. Histochem. Cytochem.* **27:** 1654-1655.

Cubero, J., Martinez, M.C., Llop, P. and Lopes, M.M. (1999) A simple and efficient PCR method for the detection of *Agrobacterium tumefaciens* in plant tumours. *Journal of Applied Microbiology* **86(4):** 591-602.

Davey, H.M., Kaprelyants, A.S. and Kell, D.B. (1993) Flow cytometric analysis, using rhodamine 123, of *Micrococcus luteus* at low growth rate in chemostat culture. In: Lloyd, D. (ed.). *Flow Cytometry in Microbiology*. London, UK: Springer-Verlag. pp. 83-93.

De Boer, S.H., Wieczorek, A. and Kummer, A. (1988) An ELISA test for bacterial ring rot with a new monoclonal antibody. *Plant Disease* **72:** 874-878.

De la Cruz, A.R., Wiese, M.V. and Schaad, N.W. (1992) A semiselective medium for isolation of *Clavibacter michiganensis* subsp. *sepedonicus* from potato tissues. *Plant Disease* **76(2):** 830-834.

Diaper, J.P., Tither, K. and Edwards, C. (1992) Rapid assessment of bacterial viability by flow cytometry. *Applied Microbiology and Biotechnology* **22:** 1-5.

Diaper, J.P., Edwards, C., Stewart-Tull, D.E.S. and Sussman, M. (1992) The detection of bacteria in aquatic environments by flow cytometry. The release of genetically modified microorganisms: In: Stewart, T.D. (ed.). *Proceedings of a symposium held under the auspices of the Federation of European Microbiological Societies. August 29-31, 1991, Nottingham, UK.* pp. 233-234.

Diaper, J.P. and Edwards, C. (1994) Flow cytometric detection of viable bacteria in compost. *FEMS Microbiology Ecology* **14:** 213-220.

Drier, J., Bermpohl, A. and Eichenlaub, R. (1995) Southern hybridization and PCR for specific detection of phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis*. *Phytopathology* **85(4):** 462-468.

Edwards, C., Diaper, J.P., Porter, J. and Pickup, R. (1993) Applications of flow cytometry in bacterial ecology. In: Lloyd, D. (ed.). *Flow Cytometry in Microbiology*. London, UK: Springer-Verlag, pp. 121-129.

Endo, H., Nakayama, J., Hayashi, T. and Watanabe, E. (1997) Application of flow cytometry for rapid determination of cell number of viable bacteria. *Fisheries Science* **63:** 1024-1029.

Engvall, E. and Perlmann, P. (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* **8:** 871-874.

Fegan, M., Croft, B.J., Teakle, D.S., Hayward, A.C. and Smith, G.R. (1998) Sensitive and specific detection of *Clavibacter xyli* subsp. *xyli*, causal agent of ratoon stunting disease of sugarcane, with a polymerase chain reaction-based assay. *Plant Pathology* **47(4):** 495-504.

Firrao, G. and Locci, R. (1994) Identification of *Clavibacter michiganensis* subsp. *sepedonicus* using polymerase chain reaction. *Canadian Journal Microbiology* **40:** 148-151.

Fraaije, B.A., Lovell, D.J., Rohel, E.A. and Hollomon, D.W. (1999) Rapid detection and diagnosis of Septoria tritici epidemics in wheat using a polymerase chain reaction/PicoGreen assay. *Journal of Applied Microbiology* **86(4):** 701-708.

Franken, A.A.J.M. (1992) Comparison of immunofluorescence microscopy and dilution-plating for the detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds. *Netherlands Journal of Plant Pathology* **98:** 169-178.

Fry, J.C. (1990) Direct methods and biomass estimation. *Methods in Microbiology* **22:** 41-85.

Gahan, P.B. (1984) Plant histochemistry and cytochemistry. Academic Press, London.

Garcia, P.M.D., Bainbridge, B.W., Heale, J.B., Perez, A.E., Jimenez, D.R.M., Pedrajas, M.D.G., Artes, E.P. and Dias, R.M.J. (1999) A simple PCR-based method for the detection of the chickpea-wilt pathogen *Fusarium oxysporum* f. sp. *ciceres* in artificial and natural soils. *European Journal of Plant Pathology* **105(3):** 251-259.

Goszczynska, T. and Serfontein, J.J. (1998) Milk-Tween agar, a semiselective medium for isolation and differentiation of *Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli*. *Journal of Microbiological Methods* **32(1):** 65-72.

Haugland, R.P. (1996) Handbook of fluorescent probes and research chemicals, 6th ed. *Molecular Probes*, Inc., Eugene, Oregon, USA, 679 pp.

Hudson, B., Upholt, W.B., Divinny, J. and Vinograd, J. (1969) The use of an ethidium bromide analogue in the dye-buoyant density procedure for the isolation of closed circular DNA: the variation of the superhelix density of mitocondrial DNA. *Proc. Natl. Acad. Sci.* USA. **62:** 813-820.

Ito, S., Maehara, T., Maruno, E., Tanaka, S., Kameya, I.M. and Kishi, F. (1999) Development of a PCR-based assay for the detection of *Plasmodiophora brassicae* in soil. *Journal of Phytopathology* **147(2):** 83-88.

Kaneshiro, E.S., Wyder, M.A., Wu, Y.P. and Cushion, M.T. (1993) Reliability of calcein acetoxy methyl ester and ethidium homodimer or propidium iodide for viability assessment of microbes. *Journal of Microbiological Methods* **17:** 1-16.

Kaprelyantes, A.S. and Kell, D.B. (1992) Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry. *Journal of Applied Bacteriology* **72:** 410-422.

Kaprelyantes, A.S. and Kell, D.B. (1993a) The use of 5-cyano-2,3-ditolyl tetrazolium chloride and flow cytometry for the visualization of respiratory activity in individual cells of *Micrococcus luteus*. *Journal of Microbiological Methods* **17:** 115-122.

Kaprelyantes, A.S. and Kell, D.B. (1993b) Dormancy in stationary phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. *Applied and Environmental Microbiology* **59:** 3187-3196.

Kishinevsky, B. and Gurfel, D. (1980) Evaluation of enzyme-linked immunosorbent assay (ELISA) for serological identification of different *Rhizobium* strains. *Journal of Applied Bacteriology* **49:** 517-526.

Kusunoki, H., Kobayashi, K., Kita, T., Tajima, T., Sugii, S. and Uemura, T.(1998) Analysis of enterohemorrhagic *Escherichia coli* serotype 0157 : H7 by flow cytometry using monoclonal antibodies. *Journal of Veterinary Medical Science* **60(12):** 1315-1319.

Lange, H., Bavouzet, J.M., Taillandier, P. and Delorme, C. (1993) Systematic error and comparison of four methods for assessing the viability of *Saccharomyces cerevisiae* suspensions. *Biotechnology Techniques* **7:** 223-228.

Langsrud, S. and Sundheim, G. (1996) Flow cytometry for rapid assessment of viability after exposure to a quartenary ammonium compound. *Journal of Applied Bacteriology* **81:** 411-418.

Leeman, M., Raaijmakers, J.M., Bakker, P.A.H.M. and Schippers, B. (1991) Immunofluorescence colony staining for monitoring pseudomonads introduced in soil. In: Beemster, A.B.R., Bollen, B.J., Gerlagh, M., Ruissen, M.A., Schippers, B. and Tempel, A. (eds.). *Biotic Interections and Soil-borne Diseases*. Amsterdam: Elsevier. pp. 374-380.

Li, H. and Walker, D.H. (1992) Characterization of rickettsial attachment to host cells by flow cytometry. *Infection and Immunity* **60(5):** 2031-2035.

Lloyd, D. and Hayes, A.J. (1995) Vigour, vitality and viability of microorganisms. *FEMS Microbiology Letters* **133:** 1-7.

Lopes, S.A. and Damann, K.E. (1997) PCR detection of *X. albilineans* from vascular sap of cane. *Sugar Cane* **5:** 13-18.

Magariños, B., Romalde, J.L., Cid, A. and Toranzo, A.E. (1997) Viability of starved *Pasteurella piscicida* in seawater monitored by flow cytometry and the effect of antibiotics on its resuscitation. *Letters in Applied Microbiology* **24:** 122-126.

Mahuku, G.S., Platt, H.W. and Maxwell, P. (1999) Comparison of polymerase chain reaction based methods with plating on media to detect and identify *Verticillium* wilt pathogens of potato. *Canadian Journal of Plant Pathology* **21(2):** 125-131.

Manulis, S., Kogan, N., Valinsky, L., Dror, O. and Kleitman, F. (1998) Detection of *Erwinia herbicola* pv. *gypsophilae* in gypsophila plants by PCR. *European Journal of Plant Pathology* **104(1):** 85-91.

Manulis, S., Kogan, N., Valinsky, L., Dror, O., Kleitman, F., Fischer, G. and Angarita, A. (1999) Detection of plant pathogens in flowers by PCR technique. *Acta Horticulturae* **482:** 117-121.

Mason, D.J., Lopes-Amoros, R., Allman, R., Stark, J.M. and Lloyd, D. (1995) The ability of membrane potencial dyes and calcafluor white to distinguish between viable and non-viable bacteria. *Journal of Applied Bacteriology* **78:** 309-315.

May, J.D., Branton, S.L., Pruett, S.B. and Ainsworth, A.J. (1994) Differentiation of two strains of *Mycoplasma gallisepticum* with monoclonal antibodies and flow cytometry. *Avian Disease* **38(3):** 542-547.

McClelland, R.G. and Pinder, A.C. (1994a) Detection of low levels of specific Salmonella species by fluorescent antibodies and flow cytometry. *Journal of Applied Bacteriology* **77:** 440-447.

McClelland, R.G. and Pinder, A.C. (1994b) Detection of *Salmonella typhimurium* in dairy products with flow cytometry and monoclonal antibodies. *Applied and Environmental Microbiology* **60(12):** 4255-4262.

McFeters, G.A., Yu, F.P., Pyle, B.H. and Stewart, P.S. (1995) Physiological assessment of bacteria using fluorochromes. *Journal of Microbiological Methods* **21:** 1-13.

McKay, G.J., Brown, A.E., Bjourson, A.J. and Mercer, P.C. (1999) Molecular characterisation of *Alternaria linicola* and its detection in linseed. *European Journal of Plant Pathology* **105(2):** 157-166.

Miyoshi, T., Sawada, H., Tachibana, Y. and Matsuda, I. (1998) Detection of *Xanthomonas campestris* pv. *citri* by PCR using primers from the spacer region between the 16S and 23S rRNA genes. *Annals of the Phytopathological Society of Japan* **64(4):** 249-254.

Muirhead, K.A., Horan, P.K. and Poste, G. (1985) Flow cytometry: Present and future. *Biotechnology* **3:** 337-356.

Muller, W. and Gautier, F. (1975) Interactions of heteroaromatic compounds with nucleic acids; A·T-specific non-intercalating DNA ligands. *Eur. J. Biochem.* **54:** 385-394.

Oh, C., Heu, S., Choi, Y., Oh, C.S., Heu, S.G. and Choi, Y.C. (1999) Sensitive and pathovar-specific detection of *Xanthomonas campestris* pv. *glycine* by DNA hybridization and polymerase chain reaction analysis. *Plant Pathology Journal* **15(1):** 57-61.

Olivier, C. and Loria, R. (1998) Detection of *Helminthosporium solani* from soil and plant tissue with species-specific PCR primers. *FEMS-Microbiology Letters* **168(2):** 235-241.

Page, S. and Burns, R.G. (1991) Flow cytometry as a means of enumerating bacteria introduced into soil. *Soil Biology and Biochemistry* **23:** 1025-1028.

Pan, Y.B., Grisham, M.P., Burner, D.M., Damann, K.E. and Wei, Q. (1998) A polymerase chain reaction protocol for the detection of *Clavibacter xyli* subsp. *xyli*, the causal bacterium of sugarcane ratoon stunting disease. *Plant Disease* **82(3):** 285-290.

Pinder, A.C., Purdy, P.W., Poulter, S.A.G. and Clark, D.C. (1990) Validation of flow cytometry for rapid enumeration of bacterial concentrations in pure cultures. *Journal of Applied Bacteriology* **69:** 92-100.

Pinder, A.C. and McClelland, R.G. (1994) Rapid assay for pathogenic *Salmonella* organisms by immunofluorescence flow cytometry. *Journal of Microscopy* **176(1):** 17-22.

Plihon, F., Taillandier, P. and Strehaiano, P. (1995) A direct and simple method for rapid counting viable chains of *Leuconostoc* in batch cultures. *Biotechnology Techniques* **9:** 451-456.

Porter, J., Pickup, R. and Edwards, C. (1997) Evaluation of flow cytometric methods for the detection and viability assessment of bacteria from soil. *Soil Biol. Biochem.* **29:** 91-100.

Porter, J., Edwards, C., Morgan, A.W. and Pickup, R.W. (1993) Rapid, automated separation of specific bacteria from lake water and sewage by flow cytometry and cell sorting. *Applied and Environmental Microbiology* **59(10):** 3327-3333.

Prosen, D., Hatzloukas, E., Schaad, N.W. and Panopoulos, N.J. (1993) Specific detection of *Pseudomonas syringe* pv. *phaseolicola* DNA in bean seed by polymerase chain reaction-based amplification of a phaseolotoxin gene region. *Phytopathology* **83:** 965-970.

Rademaker, J.L.W. and Janse, J.D. (1994) Detection and identification of *Clavibacter michiganensis* subsp. *sepedonicus* and *Clavibacter michiganensis* subsp.

michiganensis by nonradioactive hybridization, polymerase chain reaction, and restriction enzyme analysis. *Canadian Journal of Microbiology* **40:** 1007-1018.

Randhawa, P.S. and Schaad, N.W. (1984) Selective isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds. *Phytopathology* **74:** 268-272.

Rasmussen, O.F. and Wulf, B.S. (1991) Detection of *P.s.pisi* using PCR. In: Durbin, R.D., Surico, G. and Mugnai, L. (eds.). *Proceedings of International Working Group on Pseudomonas syringe pathovars, 4th Stamperia Granducale.* Florence, Italy: pp. 369-376.

Rhan, C.A., Bombick, D.W. and Doolittle, D.J. (1991) Assessment of mitochondrial membrane potential as an indicator of cytotoxicity. *Fundamental and Applied Toxicology* **16:** 435-448.

Ronot, X., Benel, L., Adolphe M. and Mounolou, J.C. (1986) Mitochondrial analysis in living cells: the use of rhodamine 123 and flow cytometry. *Biology of the Cell* **57:** 1-8.

Roth, B.L., Poot, M., Yue, S.T. and Millard, P.J. (1997) Bacterial viability and antibiotic susceptibility testing with sytox green nucleic acid stain. *Applied and Environmental Microbiology* **63:** 2421-2431.

Santos, M.S., Cruz, L., Norskov, P. and Rasmussen, O.F. (1997) A rapid and sensitive detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds by polymerase chain reaction. *Seed Science and Technology* **25:** 581-584.

Schaad, N.W. and Donaldson, R.C. (1980) Comparison of two methods for Detection of *Xanthomonas campestris* in crucifer seeds. *Seed Science and Technology* **8:** 383-391.

Schaad, N.W., Cheong, S.S., Tamaki, S., Hatziloukas, E. and Panopoulos, N.J. (1995) A combined biological and enzymatic amplification (BIO-PCR) technique to detect *Pseudomonas syringe* pv. *phaseolicola* in bean seed extract. *Phytopathology* **85:** 243-248.

Schaad, N.W., Bonde, M.R. and Hatziloukas, E. (1997) BIO-PCR: a highly sensitive technique for detecting seedborne fungi and bacteria. In: Hutchins, J.D. and Reeves, J.C. (eds.). *Seed Health Testing Progress Towards the 21st Century.* Cambridge, UK. pp. 159-164.

Schubert, R., Bahnweg, G., Nechwatal, J., Jung, T., Cooke, D.E.L., Duncan, J.M., Muller, S.G., Langebartels, C., Sandermann, H.Jr. and Osswald, W. (1999) Detection and quantification of *Phytophthora* species which are associated with

root-rot diseases in European deciduous forests by species-specific polymerase chain reaction. *European Journal of Forest Pathology* **29(3):** 169-188.

Sgorbati, S., Barbesti, S., Citterio, S., Besbetti, G. and De Vecchi, R. (1996) Characterization of number, DNA content, viability and cell size of bacteria from natural environments using DAPI/PI dual staining and flow cytometry. *Minerva Biotecnologica* **8:** 9-15.

Shapiro, H.M. (1988) Practical flow cytometry. Alan R. Liss, Inc. New York.

Shirakawa, T., Sasaki, T. and Ozaki, K. (1991) Ecology and control of tomato bacterial canker and detection methods of its pathogen. *Japan Agricultural Research Quarterly* **25(1):** 27-32.

Skowronek, P., Krummeck, G., Hoferkamp, O. and Rodd, G. (1990) Flow cytometry as a tool to discriminate respiratory-competent and respiratory-deficient yeast cells. *Current Genetics* **18:** 265-267.

Smith, O.P., Peterson, G.L., Beck, R.J., Schaad, N.W. and Bonde, M.R. (1996) Development of a PCR-based method for identification of *Tilletia indica,* causal agent of Karnal bunt of wheat. *Phytopathology* **86:** 115-122.

Steen, H.B., Boye, E., Skarstad, K., Bloom, B., Godal, T. and Mustafa, S. (1982) Applications of flow cytometry on bacteria: cell cycle kinetics, drug effects and quantitation of antibody binding. *Cytometry* **2:** 249-257.

Stevens, E.A., Blakemore, E.J.A. and Reeves, J.C. (1997) Development of a PCR-based test to detect and identify Pyrenophora ssp. In: Hutchins, J.D. and Reeves J.C. (eds.). *Seed Health Testing Progress Towards the 21st Century*. Cambridge, UK. pp. 139-145.

Syder, C.L. and Jones, A.L. (1999) Genetic variation between strains of *Monilinia fructicola* and *Monilinia laxa* isolated from cherries in Michigan. *Canadian Journal of Plant Pathology* **21(1):** 70-77.

Taylor, J.D. (1978) Detection of seed-borne bacteria. In: *Report on the Sixteenth International workshop on Seed Pathology*. pp. 28-29.

Taylor, I.W. and Milthorpe, B.K. (1980) An evaluation of DNA fluorochromes, staining techniques, and analysis for flow cytometry. I. Unperturbed cell population. *J. Histochem. Cytochem.* **28:** 1224-1232.

Thomas, J.E., Reeves, J.C., Taylor, E.J.A. and Kenyon, D.M. (1998) The development of new diagnostic techniques and their role in improving treatment strategies for seed-borne diseases. Brighton Crop Protection Conference: *Pests &*

Diseases: Volume: 3 Proceedings of an International Conference, Brighton, UK, 16-19 November 1998. pp.787-792.

Toth, I.K., Hyman, L.J., Taylor, R. and Birch, P.R.J. (1998) PCR-based detection of *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* in plant material and its differentiation from *X. c.* pv. *phaseoli*. *Journal of Applied Microbiology* **85(2):** 327-336.

Toth, I.K., Hyman, L.J. and Wood, J.R. (1999) A one step PCR-based method for the detection of economically important soft rot *Erwinia* species on micropropagated potato plants. *Journal of Applied Microbiology* **87(1):** 158-166.

Ueckert, J.E., Von-Caron, G.N., Bos, A.P. and Steeg, P.F. (1997) Flow cytometric analysis of *Lactobacillus plantarum* to monitor lag times, cell division and injury. *Letters in Applied Microbiology* **25:** 295-299.

Van Vuurde, J.W.L. and Roozen, N.J.M. (1990) Comparison of immuno-fluorescence colony-staining in media, selective isolation on pectate medium. ELISA and immunofluorescence cell staining for detection of *Erwinia carotovora* subsp. *atroseptica* and *E. chrysanthemi* in cattle manure slurry. *Netherlands Journal of Plant Pathology* **96:** 75-89.

Van Vuurde, J.W.L. and Bovenkamp, G.W. (1995) Detection of *Pseudomonas syringe* pv. *phaseolicola* in bean. In: Saettler, A.W., Schaad, N.W. and Roth, D.A. (eds). *Detection of Bacteria in Seed and Other Planting Material.* St. Paul, Minnesota, USA. pp. 30-40.

Van Vuurde, J.W.L., Kastelein, P. and Van der Wolf, J.M. (1995) Immunofluorescence colony-staining (IFC) as a concept for bacterial and ecological research. *EPPO Bulletin* **25:** 157-162.

Van Vuurde, J.W.L. (1997) Immunofluorescence colony-staining as a tool for sample indexing and for the determination on field thresholds for pathogenic seedborne bacteria. In: Hutchins, J.D. and Reeves, J.C. (eds). *Seed Health Testing · Progress Towards the 21st Century*, Cambridge U.K. pp. 165-173.

Verdier, V., Mosquera, G. and Assigbetse, K. (1998) Detection of the cassava bacterial blight pathogen, *Xanthomonas axonopodis* pv. *manihotis*, by polymerase chain reaction. *Plant Disease* **82(1):** 79-83.

Wang, Z., Yin, Y., Cheng, P., Wang, Z.K., Yin, Y.P. and Cheng. P. (1998) PCR (polymerase chain reaction) assay for identification and detection of sugarcane leaf

scald (*Xanthomonas albilineans*). *Journal of Southwest Agricultural University* **20(4):** 328-333.

Wang, Z.K., Comstock, J.C., Hatziloukas, E. and Schaad, N.W. (1999) Comparison of PCR, BIO-PCR, DIA, ELISA and isolation on semiselective medium for detection of *Xanthomonas albilineans*, the causal agent of leaf scald of sugarcane. *Plant Pathology* **48(2):** 245-252.

Widholm, J.M. (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Technology* **47:** 189-194.

Williams. S.C., Hong, Y., Danavall, D.C.A., Howard-Jones, M.H., Gibson, D., Frischer, M.E. and Verity, P.G. (1998) Distinguish between living and nonliving bacteria: evaluation of the vital stain propidium iodide and its combined use with molecular probes in aquatic samples. *Journal of Microbiological Methods* **32:** 225-236.

Chapter 2

Rapid fluorescence assessment of intracellular pH as a viability indicator of *Clavibacter michiganensis* subsp. *michiganensis*

L.G. Chitarra[1,2], P. Breeuwer[1], R.W. van den Bulk[2] and T. Abee[1]

[1]Department of Food Technology and Nutritional Sciences, Wageningen University and Research Centre, and [2]Department of Reproduction Technology, Centre for Plant Breeding and Reproduction Research (CPRO), Wageningen, The Netherlands

***Journal of Applied Microbiology* 88: 809-816. (2000)**

Summary

The viability of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) was determined by measuring the intracellular pH (pH_{in}) as a viability parameter. This was based on our observation that growth of Cmm was inhibited at pH 5.5 and below. Therefore, viable cells should maintain their pH_{in} above this pH value. The pH_{in} of Cmm was determined using the fluorescent probe 5 (and 6-)- carboxyfluorescein succinimidyl ester (cFSE). The pH_{in} of Cmm cells exposed to acid treatments was determined using fluorescence spectrofluorometry and for cells exposed to elevated temperatures, the pH_{in} was determined using fluorescence spectrofluorometry and flow cytometry (FCM). A good correlation was found between the presence of a pH gradient and the number of colony forming units observed in plate counts. However, with the spectrofluorometry technique the analysis is based on the whole cell population and the detection sensitivity of this technique is rather low, i.e. cell numbers of at least 10^7 CFU ml^{-1} are needed for the analysis. Using FCM, heat-treated and non-treated Cmm cells could be distinguished based on the absence and presence of a pH gradient, respectively. The major advantage of FCM is its high sensitivity, allowing analysis of microbial populations, even at low numbers, i.e. 10^2 - 10^3 CFU ml^{-1}.

Introduction

Clavibacter michiganensis subsp. *michiganensis* (Smith) (Davis *et al.* 1984) is a seed-transmitted plant pathogenic bacterium which causes bacterial canker of tomato (Bryan 1930). This disease is responsible for major economic losses in commercial tomato production worldwide. Seeds and plants must be free of the pathogen and in practice tomato seeds are therefore treated with hot water (Blood 1933; Shoemaker and Echandi 1976); hydrochloric acid (Thyr *et al.* 1973; Dhanvantari 1989), or sodium hypochlorite (Shoemaker and Echandi 1976). To test the efficacy of such disinfection methods, rapid, accurate and reliable methods to assess viability of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) are highly desirable.

Detection methods for Cmm which are applied to test seed lots and plants for contamination with the pathogen are usually based on immuno-fluorescence microscopy or on plating seeds or seed extracts on semi-selective agar media (Saettler *et al.* 1989). Plating methods provide information about the number of viable cells. This method, however, is time consuming. Immuno-fluorescence microscopy, on the other hand, does not discriminate between dead and live cells and is therefore not suited as a viability assay.

Various methods have been described for rapid assessment of microbial viability, including those based on assessment of cell membrane integrity with DNA probes (Kaneshiro *et al.* 1993; Bunthof *et al.* 1999), and the capability of cells to maintain a membrane potential as determined by probe uptake or exclusion (Kaprelyants and Kell 1992; Mason *et al.* 1995). Additionally, the capability of cells to maintain a pH gradient (pH_{in} higher than pH_{out}) may also supply information about viability. The pH_{in} was successfully determined in several gram positive bacteria using the pH dependent fluorescent probe 5 (and 6-)-carboxyfluorescein succinimidyl ester (cFSE) (Breeuwer *et al.* 1996). cFSE can form conjugates with aliphatic amines and is therefore better retained within the cell than non-conjugated probes such as carboxyfluorescein (cF) and 2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) (Haugland 1992; Breeuwer *et al.* 1996).

In this study, cFSE was tested for determination of the pH_{in} of Cmm and subsequently this parameter was evaluated for its use as a viability indicator. The pH_{in} of Cmm exposed to stress conditions, such as elevated temperatures and acid treatments, was determined at the population level with cell suspensions containing at

least 10^7 CFU ml^{-1} using fluorescence spectrofluorometry, and at the level of individual cells using flow cytometry. FCM has been used successfully to distinguish between viable and non-viable bacteria after staining with a range of fluorescent probes such as cFDA, Calcein AM and Rhodamine 123 (Diaper and Edwards 1994). The results obtained with the fluorescence techniques were compared with the conventional plate count technique.

Materials and Methods

Growth conditions and determination of growth curves

Clavibacter michiganensis subsp. *michiganensis* (Cmm) NCPPB 1064 was grown on 1% Glucose-Nutrient-Agar (GNA; Oxoid) medium for 48 hours at 25°C. For growth in liquid medium, Cmm cells were cultured in Nutrient Broth (NB; Oxoid) or in NB supplemented with 10 g l^{-1} glucose (GNB), pH 7.2, on a rotary shaker (180 rev min^{-1}) at 25°C for 24 hours. For determination of growth curves, the optical density (O.D.) of 3 replicate samples containing the bacterial cells was measured every 2 hours with a spectrophotometer at 620 nm ($O.D._{620}$) for a period of 30 hours. The initial $O.D._{620}$ was approximately 0.06.

Bacterial cells were also grown in GNB medium supplemented with 200 mM of potassium chloride (KCl) at different pH values. The initial $O.D._{620}$ of three replicate samples of 250 µl containing the bacterial cells was approximately 0.04. The ionophore nigericin at a final concentration of 0.1 µmol l^{-1} was added to each sample, except for the control samples, and the $O.D._{620}$ was measured every 15 minutes with a multititer plate reader (SLT Labinstrument G.M.B.H., Crailsheim, Germany) for a period of 12 hours.

Plate count technique

Plate counts were determined as described by Miles and Misra (1933). Serial dilutions were made from each bacterial suspension and 20 µl drops of each dilution were plated in triplicate on sectored GNA plates. After incubation of plates for 48 hours at 25°C, the number of colonies was counted for those dilutions producing between 3 and 30 colonies per 20 µl drop, and the total viable counts for different treatments were calculated.

Temperature and acid treatment

Bacterial cells grown in GNB medium at 25°C for 10 hours were harvested at mid-exponential phase ($O.D._{620}$ approximately 0.4). Cells grown for 48 hours at 25°C on GNA medium were collected at stationary phase by washing off the cells in CPK buffer containing citric acid (50 mmol l^{-1}), $Na_2HPO_4 \cdot 2H_2O$ (50 mmol l^{-1}) and KCl (50 mmol l^{-1}), pH 5.5 or 7.0. The cell suspension was adjusted to an $O.D._{620}$ of approximately 0.4. The harvested cells were incubated at 25 (control), 40, 45 or 50°C, or in buffer without (control) and with 0.1, 0.2 or 0.6 mol l^{-1} HCl for 1 hour. After the treatment, the cells were spun down (15000*g* for 3 minutes), resuspended in CPK buffer pH 5.5 or 7.0 and stored on ice until required.

Labeling of cells with fluorescent probe

Cmm cells in CPK buffer were incubated for 15 minutes at room temperature in the presence of 1.0 µM of 5 (and 6-)-carboxyfluorescein diacetate succinimidyl este (cFDASE), washed twice, resuspended in CPK buffer and incubated for 15 minutes with glucose (10 mmol l^{-1}). Subsequently, the cells were washed, resuspended in buffer, and again glucose (10 mmol l^{-1}) was added to the cells. Finally, the cells were washed and resuspended in CPK buffer, pH 5.5 or 7.0, and placed on ice until required.

Determination of pH_{in}

The pH_{in} of Cmm was analysed according to the method of Breeuwer *et al.* (1996) with some modifications. Cmm cells containing fluorescent probe were diluted to a concentration of approximately 10^8 cells ml^{-1} in a 3-ml glass cuvette and placed in the stirred and thermostated cuvette holder of the spectrofluorometer (Perkin Elmer LS 50B, Norwalk, UK). Fluorescence intensities were measured at excitation wavelengths of 500 and 440 nm by rapidly altering the monochromator between both wavelengths. The emission wavelength was 530 nm, and the excitation and emission slit widths were 5 and 10 nm, respectively. The incubation temperature was 25°C. At the end of each assay the extracellular fluorescence signal (background) was determined by filtration of the cell suspension through a 0.22 µm pore-size membrane filter and measuring the cell-free filtrate. The 500-to-440 nm ratios were corrected for these background signals.

Calibration curves of cFSE loaded cells at different pH_{in} values were determined in CPK buffer with pH values ranging from 4.0 to 8.0 adjusted with HCl. The fluorescence intensity was measured at 25°C after equilibrating pH_{in} and pH_{out} by addition of valinomycin (1 µmol l^{-1}) and nigericin (1 µmol l^{-1}). The calibration curve was fitted according to a 4 parameter sigmoid function $y=a+b/\{1+\exp[-(x-c)/d]\}$ and the parameters a to d were determined. The pH_{in} was calculated using the formula $pH_{in}=-[\ln((b-(ratio-a))/(ratio-a))\ x\ d] + c$.

Flow cytometric analysis of the intracellular pH

Analysis of individual cells was performed with a FACSCalibur flow cytometer (Becton-Dickinson Benelux N.V., Erembodegem, Belgium), equipped with an air-cooled argon ion laser (excitation wavelength 488 nm), which was operated at 15 mW. The instrument was set up to collect 6 parameters: forward and side scatter, fluorescent light at emission wavelengths of 530±15 nm (FL-1; green fluorescence), 585±21 nm (FL-2; orange fluorescence), and > 670 nm (FL-3; red fluorescence), and time. The low angle light scatter (forward scatter) and the wide angle light scatter (side scatter) were used as indicators of cell size and granularity, respectively. The results are represented in 2 parameter dot plots in which the X-axis and Y-axis are divided into 1024 channels, relative to the intensity of the incoming signal. A logarithmic amplification of the incoming signal was used to measure a wider range of signals (4 decade log scale). The sample analysis time and the flow rate were approximately 2 minutes and 12 µl min^{-1}, respectively. The cells were separated from background by their side and forward scatter characteristics. From the FL1/FL2 dot plots the ratio of the green and the orange signals (FL1/FL2) could be calculated (after back transformation from log to linear mode). From this ratio the intracellular pH could be calculated.

Results

Growth curves under various conditions

Growth of Cmm was determined in NB without and with glucose (GNB). The presence of glucose resulted in faster growth and higher cell densities. Since GNB showed a more pronounced exponential phase compared to NB, it was selected as the standard growth medium for further experiments (Fig.1).

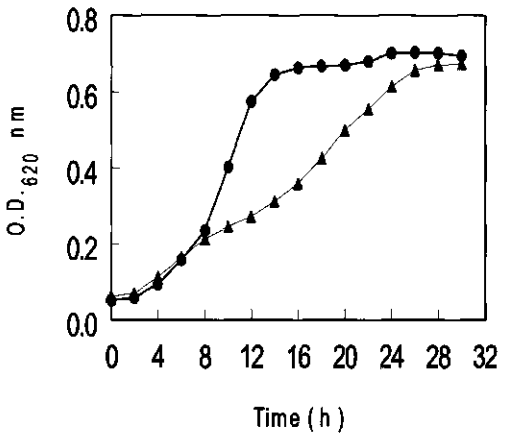

Fig. 1. Growth curve of *Clavibacter michiganensis* subsp. *michiganensis* in Nutrient Broth (NB) medium without (▲) and with 10g l^{-1} glucose (•). Cells were grown on a rotary shaker (180 rev min^{-1}) at 25°C for 30 hours.

Additionally, it allows the use of glucose as an energy source in the in vitro experiments. Exponential phase cells ($O.D._{620}$ of suspension in GNB approximately 0.4) and stationary phase cells (cells harvested from GNA after 48 hours with $O.D._{620}$ of the suspension adjusted to 0.4) were used in the experiments.

Growth of Cmm cells in GNB medium supplemented with KCl in the absence and presence of nigericin was evaluated to determine the minimum pH_{in} value at which cells are able to grow (Fig. 2).

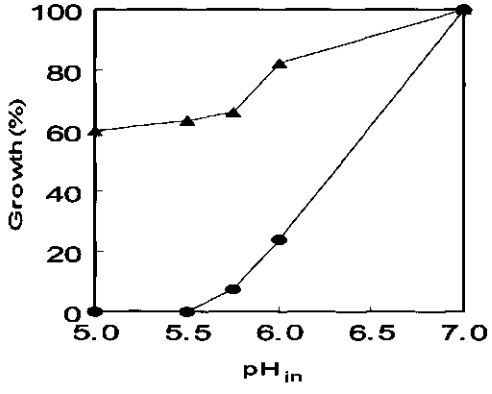

Fig. 2. Growth of *Clavibacter michiganensis* subsp. *michiganensis* in GNB medium supplemented with 200 mmol l^{-1} of KCl without (▲) and with 0.1 µmol l^{-1} of nigericin (•). The bacteria were incubated in GNB medium with pH varying from 5.0 to 7.0, at 25°C for 12 hours. Growth was expressed as percentage of maximum growth at pH 7.0.

Maximum growth was observed at pH 7.0. Growth decreased with a decrease of medium pH, with 60% growth at pH 5.0 compared to that at pH 7.0. In the presence of nigericin (0.1 μmol^{-1}), which equilibrates the intracellular and the extracellular pH, Cmm was not able to grow at pH 5.5 and below. This sets the minimal intracellular pH allowing growth of Cmm at pH 5.5.

Calibration of pH_{in} measurements

Calibration curves with cFSE loaded Cmm cells were determined to enable calculation of pH_{in} values from fluorescence ratios. The ratios of the pH-sensitive wavelength (500 nm) and the pH-insensitive wavelength (440 nm) versus pH_{in} were determined in CPK buffer with pH varying from 4.0 to 8.0. For each treatment, e.g. different growth conditions or exposure to different temperatures, calibration curves were determined (Fig. 3).

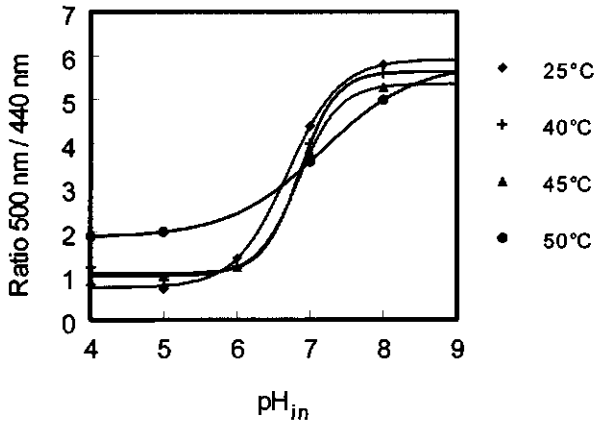

Fig. 3. The relationship between the intracellular pH (pH_{in}) and the fluorescence ratio (500 nm/440 nm)of cFSE labelled *Clavibacter michiganensis* subsp. *michiganensis* pre-cultured in GNB medium and treated at different temperatures. The pH_{in} and pH_{out} were equilibrated by adding valinomycin (1 $\mu mol\ l^{-1}$) and nigericin (1 $\mu mol\ l^{-1}$).

The results of pH_{in} measurements for non-treated cells and for cells exposed for 1hour at 45°C in buffer pH 7.0, are shown in Figure 4. After addition of glucose to energize the cells, the ratio 500 nm/440 nm showed a slight increase for non-treated cells (pH_{in} 7.3). The addition of the K^+ ionophore valinomycin resulted in dissipation of the membrane potential (negative inside) which is compensated by an increase in

the pH gradient, i.e. pH_{in} increased to 7.8. Subsequently, addition of nigericin dissipated the pH gradient ($pH_{in} = pH_{out}$ = pH 7.0).

For heat-treated Cmm cells, no pH gradient could be observed, neither after the addition of glucose nor after the addition of valinomycin and nigericin.

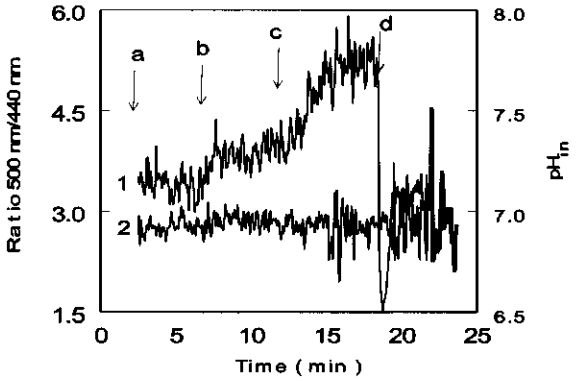

Fig. 4. Intracellular pH of Cmm control cells (1) and treated at 45°C for 1 hour (2) in CPK buffer at pH 7.0. The cells were loaded with 1.0 µM cFDASE. Measurements in the cuvette were performed at 25°C in 3 ml of CPK buffer pH 7.0. The following additions were made at the time indicated by the arrows: a, cell suspension (200 µl); b, glucose (10 mmol l^{-1}); c, valinomycin (1 µmol l^{-1}); and d, nigericin (1 µmol l^{-1}).

Effect of high temperature treatment on the pH_{in} of *Clavibacter michiganensis* subsp. *michiganensis*

cFSE-labeled Cmm cells, grown in GNB or on GNA, were incubated for 1 hour at different temperatures (25, 40, 45 and 50°C), and in CPK buffer of pH 5.5 and 7.0 for pH_{in} measurements. The results (Table 1) show that at increased temperatures, both the capacity to maintain a pH gradient and the number of colony-forming units decreased.

Control cells and cells treated at 40°C were able to maintain a pH gradient. However, both the gradient and the recovery of Cmm cells after plating showed a decrease for the 40°C treated cells compared to the control. No pH gradient was observed for cells treated at 45 and 50°C for 1 hour and cells could not, or only in low numbers be recovered after plating. Cells incubated in buffer of pH 5.5 always showed a higher pH gradient than when incubated in buffer of pH 7.0.

A good correlation ($r^2 \geq 0.80$) was found between the number of CFU ml^{-1} determined by plate counting and the magnitude of the pH gradient determined for the whole population provided the log reduction of the plate counts is less than 3.

Table 1. The effect of temperature treatment on the intracellular pH of *Clavibacter michiganensis* subsp. *michiganensis*. The bacteria were grown in NB liquid medium supplemented with 10g l^{-1} glucose (GNB) or on NA medium, also supplemented with 10g l^{-1} glucose (GNA). Cells in CPK buffer pH 5.5 or 7.0 wereexposed to different temperatures for 1 hour. Cells were loaded with cFSE and the pH_{in} measurements were done as described in Materials and Methods.

Growth medium	pH of buffer	Temperature (°C)	pH_{in} + glucose ($10mmol^{-1}$)	pH gradient (pH_{in} - pH_{out})	Plate counts (CFU ml^{-1})	Reduction log units
GNB	5.5	25	6.6	1.1	$1.28x10^9$	0
		40	6.4	0.9	$3.16x10^7$	1.6
		45	5.5	0	0	9.1
		50	5.5	0	0	9.1
	7.0	25	7.2	0.2	$1.40x10^9$	0
		40	7.15	0.15	$3.66x10^7$	1.73
		45	7.0	0	0	9.29
		50	7.0	0	0	9.29
GNA	5.5	25	6.5	1.0	$1.05x10^9$	0
		40	5.9	0.4	$2.16x10^7$	1.69
		45	5.5	0	0	9.02
		50	5.5	0	0	9.02
	7.0	25	7.5	0.5	$1.63x10^9$	0
		40	7.2	0.2	$3.33x10^6$	2.69
		45	7.0	0	$3.83x10^2$	6.63
		50	7.0	0	0	9.21

Effect of HCl treatment on the pH_{in} of *Clavibacter michiganensis* subsp. *michiganensis*

Cmm cells were shown to be very sensitive to acid (HCl) treatment. A pH gradient could not be observed for any of the treated cell suspensions (Table 2). The ratio of fluorescence intensities at 500 nm and 440 nm of cFSE stained treated cells was constant, even after the addition of glucose, or valinomycin and nigericin (pH_{in} = pH_{out}). This suggests that the cells were not viable (no pH gradient). Observations by fluorescence microscopy showed that, as the concentration of HCl increased, the intensity of fluorescence of stained cells decreased. In plate count assays, the percentage of viable cells was strongly reduced and the difference with the control value, expressed as the reduction in log unit, showed that only a very small percentage (< 0.001%) of the population could be recovered after the 0.1 mol l^{-1} HCl treatment.

Table 2. The effect of acid treatment (HCl) on the intracellular pH of *Clavibacter michiganensis* subsp. *michiganensis*. The bacteria were grown in NB liquid medium supplemented with 10g l^{-1} glucose (GNB) or on NA medium, also supplemented with 10g l^{-1} with glucose (GNA). Cells were incubated in HCl at different molaritiesfor 1 hour and then resuspended in CPK buffer pH 5.5 or 7.0. Cells were loaded with cFSE and the pH_{in} measurements were done as described in Materials and Methods.

Growth medium	pH of buffer	HCl treatment (mol l^{-1})	pH_{in} +glucose (10mmol l^{-1})	pH gradient (pH_{in} - pH_{out})	Plate counts (CFU ml^{-1})	Reduction log units
GNB	5.5	0	6.8	1.3	$1.85x10^9$	0
		0.1	5.5	0	$8.03x10^2$	6.33
		0.2	5.5	0	$4.40x10^1$	7.59
		0.6	5.5	0	0	9.23
	7.0	0	7.3	0.3	$2.96x10^9$	0
		0.1	7.0	0	$1.68x10^4$	5.24
		0.2	7.0	0	0	9.47
		0.6	7.0	0	0	9.47
GNA	5.5	0	7.1	1.6	$6.68x10^9$	0
		0.1	5.5	0	0	9.82
		0.2	5.5	0	0	9.82
		0.6	5.5	0	0	9.82
	7.0	0	8.1	1.1	$6.00x10^9$	0
		0.1	7.0	0	$1.71x10^3$	6.55
		0.2	7.0	0	0	9.78
		0.6	7.0	0	0	9.78

Flow cytometric analysis of the intracellular pH of Cmm cells

The spectrofluorometry technique allows rapid measurement at the population level, however, at least 10^7 viable cells ml^{-1} are needed to be able to detect a pH gradient. FCM allows measurement of individual cells and it detects low numbers of cells i.e. approximately 10^2 - 10^3 CFU ml^{-1}.

Fig. 5 (A) shows the results of the flow cytometric analysis of the FL1/FL2 ratio for non-treated and heat-treated Cmm cells (40°C for 1 hour) in CPK buffer at pH 5.5. For non-treated cells in the presence of nigericin (c), the mean value of the emission ratio (FL1/FL2) of fluorescence (ERF) is 1.254, which corresponds to an intracellular pH of 5.5 (no pH gradient). For cells treated at 40°C (b), the mean value of ERF is 1.291, which is close to the ratio (pH_{in}) of the ionophore treated cells. A higher pH_{in} was observed for non-treated cells (a), which showed a mean value of ERF of 1.702.

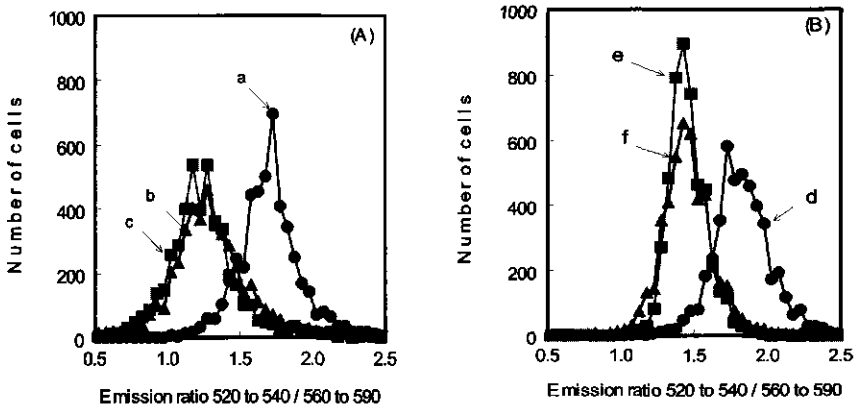

Fig. 5. Flow cytometric analysis of the intracellular pH of Cmm cells incubated in CPK buffer at pH 5.5 (A) and at pH 7.0 (B). Cells were loaded with 1.0 µmol l^{-1} cFDASE. The following treatments were given to the bacterial cells: a and d, non-treated (control); b and f, cells treated at 40°C; e, cells treated at 45°C; and c, non-treated cells in the presence of nigericin (0.1 µmol l^{-1}).

Fig. 5 (B) shows non-treated Cmm cells and cells treated at 40 or 45°C for 1 hour, in CPK buffer at pH 7.0. For cells treated at 40 (f) and 45°C (e), the mean value of ERF is 1.453, which corresponds to an intracellular pH of 7.0. Treated cells, thus, show no pH gradient. For non-treated cells (d), the mean value of ERF was significantly higher, i.e. 1.821, indicating the presence of a pH gradient.

The results show that the flow cytometric analysis allowed distinguishing between heat-treated and non-treated Cmm cells based on differences in their pH gradients.

Discussion

In this study, fluorescence assessment of pH_{in} of *Clavibacter michiganensis* subsp. *michiganensis* was used as an indicator for viability of Cmm. This application is based on the capability of a cell to maintain its pH gradient at conditions where the external pH is suboptimal, i.e. pH_{in} higher than pH_{out}. It was shown for Cmm that when the intracellular pH drops to 5.5 or below, growth was inhibited. Therefore, viable cells should maintain their pH_{in} above this pH value. Studies with *Leuconostoc mesenteroides* and *Lactobacillus plantarum* showed that these microorganisms were not able to grow when their pH_{in} dropped below 5.4 and 4.6, respectively (McDonald *et al.* 1990).

The decrease in the ability of Cmm cells to maintain a pH gradient indicate that the temperature treatments affected the viability of Cmm cells as demonstrated by the decrease in the number of CFUs observed in the plate count assay. The viability of bacterial cells was also affected when the cells were treated with HCl at different molarities (0.1, 0.2 or 0.6 mol l^{-1}). In HCl treated cells no pH gradient could be detected (pH_{in} = pH_{out}). Fluorescence microscopy revealed that these cells were poorly labeled with cFSE, either due to a low esterase activity in the cytoplasm or due to an increased efflux of cFSE resulting from the cell membrane damage caused by the acid treatment. The spectrofluorometer analysis for pH_{in} measurements was not able to detect the signal of these weakly stained cells and only a small percentage of the HCl treated cells (<0.001%) could be recovered on plate. Although, the spectrofluorometry technique allows rapid measurement at the population level, at least 10^7 viable cells ml^{-1} are needed to be able to detect a pH gradient, i.e to get a fluorescence ratio signal of sufficient magnitude. This indicates that the sensitivity of this technique is rather low.

FCM allows analysis of individual cells based on their physical and/or chemical characteristics such as size, granularity and DNA content. A major advantage of using FCM are its sensitivity and its capability to detect low number of cells, i.e 10^2 - 10^3 cells ml^{-1}. Here we showed that populations of heat-treated and non-treated Cmm cells could be distinguished based on the differences in the fluorescence ratios (pH gradients) after labeling with cFSE. The heat-treated cells had a low fluorescence ratio (no pH gradient) and could not be recovered on plates, whereas the ratio of live cells was significantly higher (pH gradient present). FCM was used successfully before to distinguish between viable and non-viable bacteria after staining with a range of fluorescent probes, such as cFDA, Calcein AM and Rhodamine 123 (Diaper and Edwards 1994; Magariños *et al.* 1997; Porter *et al.* 1997).

Overall, a good correlation was found between the presence of a pH gradient, determined by applying the fluorescent probe cFSE, and the number of colony forming units observed in plate counts. Compared to the conventional plating assays, which for Cmm takes at least 4 - 5 days, the use of FCM seems promising, especially for fundamental studies with pure cultures. For determining viability of Cmm in an environment with other saprophytic microorganisms specific detection of Cmm should be achieved as well. This is the topic of further studies.

Acknowledgement

We thank the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES (Brasil) for providing financial support to L. G. Chitarra during this study.

References

Blood, H.L. (1933) The control of tomato bacterial canker (*Aplanobacter michiganensis* E.F.S.) by fruit pulp fermentation in the seed extraction process. *Proc. Utah Acad. Sci. Arts and Letters* **10:** 19-23.

Breeuwer, P., Drocourt, J.L., Rombouts, F.M. and Abee, T. (1996) A novel method for continuous determination of the intracellular pH in bacteria with the internally conjugated fluorescent probe 5 (and 6-)-carboxyfluorescein succinimidyl ester. *Applied and Environmental Microbiology* **62:** 178-183.

Bryan, M.L. (1930) Studies on bacterial canker of tomato. *Journal of Agricultural Research* **41:** 825-851.

Bunthof, C.J., Braak, S., Breeuwer, P., Rombouts, F.M. and Abee, T. (1999) Rapid fluorescence assessment of the viability of stressed *Lactococcus lactis*. *Applied and Environmental Microbiology* **65:** 3681-3689.

Davis, M.J., Gillaspie, A.G., Jr., Vidaver, A.K. and Harris, R.W. (1984) *Clavibacter*: A new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *xyli* sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *cynodontis* subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and bermudagrass stunting disease. *Int. J. Syst. Bacteriol.* **34:**107-117.

Dhanvantari, B.N. (1989) Effect of seed extraction methods and seed treatments on control of tomato bacterial canker. *Canadian Journal of Plant Pathology* **11:** 400-408.

Diaper, J.P. and Edwards, C. (1994) The use of fluorogenic esters to detect viable bacteria by flow cytometry. *Journal of Applied Bacteriology* **77:** 221-228.

Haughland, R.P. (1992) In: Larison, K.D. (ed.). *Handbook of fluorescent probes and research chemicals*. Molecular Probes Inc., Eugene, USA, 421 pp.

Kaneshiro, E.S., Wyder, M.A., Wu, Y.P., and Cushion, M.T. (1993) Reliability of calcein acetoxy methyl ester and ethidium homodimer or propidium iodide for viability assessment of microbes. *Journal of Microbiological Methods* **17:** 1-16.

Kaprelyants, A.S and Kell, D.B. (1992) Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry. *Journal of Applied Bacteriology* **72:** 410-422.

Magariños, B., Romalde, J.L., Cid, A. and Toranzo, A.E. (1997) Viability of starved *Pasteurella piscicida* in seawater monitored by flow cytometry and the effect of antibiotics on its resuscitation. *Letters in Applied Microbiology* **24(2):** 122-126.

Mason, D.J., Lopez-Amoros, R., Allman, R., Stark, J.M. and Lloyd, D. (1995) The ability of membrane potential dyes and calcafluor white to distinguish between viable and non-viable bacteria. *Journal of Applied Bacteriology* **78:** 309-315.

McDonald, L.C., Fleming, H.P., Hassan, H.M. (1990) Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum. Applied and Environmental Microbiology* **56(7):** 2120-2124.

Miles, A.A and Misra, S.S. (1933) The estimation of the bacterial power of the blood. *The Journal of Hygiene* **38:** 732-749.

Porter, J., Pickup, R. and Edwards, C. (1997) Evaluation of flow cytometric methods for the detection and viability assessment of bacteria from soil. *Soil Biology and Biochemistry* **29(1):** 91-100.

Schaad, N.W.(1989) Detection and identification of bacteria. In: Saettler, A.W., Schaad, N.W. and Roth, D.A. (eds.). *Detection of bacteria in Seed and Other Planting Material.* St Paul, Minnesota: The American Phytopathological Society. pp. 9-16.

Shoemaker, P.B. and Echandi, E. (1976) Seed and plant bed treatments for bacterial canker of tomato. *Plant Dis. Rep.* **60:** 163-166.

Thyr, B.D., Webb, R.E., Jaworski, C.A. and Ratcliffe, T.J. (1973) Tomato bacterial canker: Control by seed treatment. *Plant Dis. Rep.* **57:** 974-977.

Chapter 3

The use of fluorescent probes to assess viability of the plant pathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* by Flow Cytometry

L.G.Chitarra [1,2], P. Breeuwer[2], T. Abee[2] and R. W. van den Bulk[1]

[1]Plant Research International, Business Unit Plant Development and Reproduction, Wageningen, The Netherlands, and [2]Department of Food Technology and Nutritional Sciences, Wageningen University, Wageningen, The Netherlands

Submitted to *Journal of Applied Microbiology*

Summary

The determination of the viability of bacteria by the conventional plating techniques is a time-consuming process. Methods based on enzyme activity or membrane integrity are much faster and may be good alternatives. Assessment of the viability of suspensions of the plant pathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) using the fluorescent probes Calcein acetoxy methyl ester (Calcein AM), carboxyfluorescein diacetate (cFDA), and propidium iodide (PI) in combination with flow cytometry was evaluated.

Heat-treated and viable (non-treated) Cmm cells labeled with Calcein AM, cFDA, PI, or combinations of Calcein AM and cFDA with PI could be distinguished based on their fluorescence intensity in flow cytometry analysis. Non-treated cells showed relatively high green fluorescence levels, whereas damaged cells (heat-treated) showed high red fluorescence levels. Flow cytometry allowed a rapid quantification and separation of viable Cmm cells labeled with Calcein AM or cFDA and heat-treated cells labeled with PI. Therefore, the application of flow cytometry in combination with fluorescent probes appears to be a promising technique for assessing viability of Cmm cells in suspensions when cells are labeled with Calcein AM or the combination of Calcein AM with PI.

Introduction

Bacterial canker of tomato, caused by the seed-transmitted plant pathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* (Smith) (Davis *et al.* 1984), causes major economic losses in commercial tomato production worldwide. To prevent the introduction of bacterial canker, disease-free seeds should be used. Indexing seeds for the presence of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is recommended to select for disease-free seeds. Information on the viability of the target bacterium is essential for decision-making, but also to determine the effect of seed treatments. Therefore, accurate and rapid methods to assess the viability of plant pathogenic bacteria, such as Cmm, are highly desirable.

Here we define viability as the capability of a cell to perform all the necessary functions for its survival under given conditions. Viable microorganisms require an intact cytoplasmic membrane, DNA transcription and RNA translation, enzyme activity, and growth (Breeuwer *et al.* 1996). The plate count method is commonly applied for detection and enumeration of viable bacteria and involves plating serial dilutions of an extract or washing on a selective medium. Subsequently, the isolated organisms may be identified by a range of biochemical and serological tests. However, the plate count method is time consuming (Plihon *et al.* 1995) and the results are sometimes difficult to interpret due to cell concentration effects, interference by other microorganisms, or presence of inhibitory components.

The development of fluorescent probes for cellular functions has led to new techniques for measuring the viability of microorganisms (Haugland 1996). Various fluorescent probes have the ability to explore different properties of the cell, such as enzyme activity (Kaneshiro *et al.* 1993; Diaper and Edwards 1994a, 1994b; Endo *et al.* 1997; Ueckert *et al.* 1997; Bunthof *et al.* 1999); cytoplasmic membrane permeability (Magariños *et al.* 1997; Porter *et al.* 1997; Roth *et al.* 1997; Williams *et al.* 1998), membrane potential (Kaprelyants and Kell 1992; Mason *et al.* 1995; Langsrud and Sundheim 1996), respiratory activity (Kaprelyants and Kell 1993a, 1993b); relative DNA content (Allman *et al.* 1992; Christensen *et al.* 1993; Sgorbati *et al.* 1996; Bernander *et al.* 1998) and pH gradient (Breeuwer *et al.* 1996; Chitarra *et al.* 2000). Several of these parameters can be used as viability indicators.

One of the enzyme activity probes that has been used as a cell viability indicator is fluorescein diacetate (FDA). FDA is a non-fluorescent polar ester

compound that can permeate intact cell membranes. Once inside the cell it is cleaved (hydrolysed) by non-specific esterases to release fluorescein, a polar fluorescent compound which is retained inside the cells. Thus, the viability can be correlated with the ability of the cell to accumulate fluorescein due to esterase activity (Widholm 1972). However, fluorescein is poorly retained by viable cells (Fry 1990; Edwards *et al.* 1993), and the FDA method can be easily frustated due to leakage or active efflux of fluorescein to the extracellular environment. This first problem can be minimized by using probes which are more negatively charged at physiological pH, resulting in less leakage of fluorescein from the cells. Such probes are for instance carboxyfluorescein diacetate (cFDA) and calcein acetoxy methyl ester (Calcein AM). To prevent active extrusion, the assay has to be performed with de-energized cells or in buffer without energy source or on a very short timescale.

Propidium iodide (PI) is a nucleic acid probe that has also been used successfully to assess viability of microorganisms (Sgorbati *et al.* 1996; Magariños *et al.* 1997). PI is not capable of crossing intact membranes of living microorganisms (Alvarado-Aleman *et al.* 1996), but it passes through damaged cell membranes. Once inside the cell, PI intercalates into RNA and DNA backbones independently of base pair ratio (Taylor and Milthorpe 1980) and A·T-rich regions (Crissman *et al.* 1979). It can also form complexes with double-stranded DNA and RNA (Hudson *et al.* 1969). PI-stained cells are assumed to be non-viable.

The potential of such viability measurements is increased when applied in combination with flow cytometry (FCM), a technique highly suited for the rapid analyses of fluorescent cells.

The aim of the present paper was to test the enzyme activity probes cFDA and Calcein AM, and the nucleic acid probe PI in combination with FCM, to determine the viability of Cmm cells. The results are compared with those of the conventional plate count technique.

Materials and Methods

Growth conditions

Clavibacter michiganensis subsp. *michiganensis* (Cmm) NCPPB 1064 was grown on 1% Glucose-Nutrient-Agar (GNA; Oxoid) medium for 24 hours at 25°C. The cells were harvested and resuspended in 0.2 M sodium phosphate buffer (SP_i) pH 7.4. The

Optical Density (O.D.) was measured with a spectrofotometer at 620 nm and adjusted by diluting with SP_i to approximately 0.35, in order to obtain concentrations of 10^6 to 10^7 colony forming units per ml (CFU ml^{-1}).

Temperature treatment

To obtain non-viable bacterial cells, the bacterial suspensions were heat-treated (Test Tube Heater SHT 2D) at 80°C for 30 minutes.

Non-treated and heat-treated bacterial cells were mixed in different ratios, i.e. 100/0, 80/20, 50/50, 20/80, and 0/100% respectively, to create populations varying in viability.

Labeling of cells with fluorescent probes

Cmm cells in SP_i pH 7.4 were incubated for 1 hour at 28°C in the presence of cFDA (10 μM), Calcein AM (10 μM), or in combination with PI (10 μM) for double labeling. Samples to be stained with PI were incubated for 20 minutes at room temperature. Subsequently, the cells were spun down at 11000*g*, washed, resuspended in SP_i pH 7.4, and placed on ice until required.

Flow cytometric analysis

Analysis of individual cells was performed with a FACSCalibur flow cytometer (Becton-Dickinson Benelux N.V., Erembodegem, Belgium), equipped with an air-cooled argon ion laser (excitation wavelength 488 nm), which was operated at 15 mW. The instrument was set up to collect 6 parameters: forward and side scatter, fluorescent light at emission wavelengths of 530/30 nm (FL-1; green fluorescence), 585/42 (FL-2; orange fluorescence), and > 670 nm (FL-3; red fluorescence), and time. The low angle light scatter (forward scatter) was used as an indicator of cell size and the wide angle light scatter (90° or side scatter) was used as an indicator of cell granularity. The results are represented in 2 parameter dot plots in which the X-axis and Y-axis are divided into 1024 channels, relative to the intensity of the incoming signal. A logarithmic amplification of the incoming signal was used to measure a wider range of signals (4 decade log scale). The sample analysis time was approximately 2 minutes and the number of labeled bacterial cells was quantified based on the flow rate, which was determined to be 4.8 μl min^{-1}. The cells were

separated from the background by their side scatter characteristics. Non-treated (viable) unstained cells were used as negative controls for Calcein AM and cFDA, and heat-treated (non-viable) unstained cells for PI.

To verify that green and red fluorescent cells represent live (non-treated) and dead (heat-treated) bacterial cells, respectively, cells from defined populations were sorted and plated.

Sorting of viable and non-viable cells

Cmm cells labeled with Calcein AM, cFDA or PI were sorted based on the green and red fluorescence of the cells at 530/30 and > 670 nm respectively. Calcein AM and cFDA positive cells give high green fluorescence signal (viable cells), and PI positive cells give a high red fluorescence signal (non-viable cells). Cells from these two distinct fluorescence regions were sorted, counted by the flow cytometer, and 50 µl drops containing sorted viable or non-viable cells were plated on GNA medium. After incubation of plates for 96 hours at 25°C, the number of colonies was counted and the total number of CFU ml^{-1} was calculated. Sorted cells were also counted in a Neubauer counting chamber.

Plate count technique

Plate counts were determined as described by Miles and Misra (1933). Serial ten-fold dilutions were made from each bacterial suspension in SP_i pH 7.4, and 20 µl drops of each dilution were plated in triplicate on sectored GNA plates. After incubation of plates for 72h at 25° C, the number of colonies was counted for those dilutions producing between 3 and 30 colonies per 20 µl drop, and the total number of CFU ml^{1} was calculated for each sample.

Total cell counts

Four replicates of each sample were counted in a Neubauer counting chamber with a fluorescence microscope (Zeiss, Axiophoto, West Germany) at a magnification of 10 × 40. The total count determination was done by counting the non-treated cells stained with Calcein AM or cFDA (green fluorescence), and heat-treated cells stained with PI (red fluorescence). Non-labeled cells were counted as well (control).

Results

For the FCM-based viability assay, Cmm cells were labeled with Calcein AM, cFDA, PI, or combinations of Calcein AM and cFDA with PI and analysed. The number of green and red fluorescent particles, labeled with Calcein AM, cFDA or PI was quantified. Green particles represented cells with intact membranes and esterease activity, whereas red particles represented cells with a damaged membrane (Fig. 1).

Fig. 1. Fluorescence microscopy of Cmm cells labeled with Calcein AM (green fluorescence) and PI (red fluorescence).

The FCM analyses showed that individual cells labeled with Calcein AM or cFDA, and cells labeled with PI could be perfectly separated into two well distinct regions based on their fluorescence intensity, as shown for instance for Cmm population containing 50% heat-treated cells (Fig. 2). Non-treated Calcein AM-stained cells showed relatively high green fluorescence levels (R2), whereas damaged cells (heat-treated) showed high red fluorescence levels (R3) when stained with PI.

The quantitative results of the FCM analyses were compared with total counts and plate counts for populations containing 100, 80, 50, 20 and 0% viable Cmm cells. The total counts were all in the same range, i.e. 2.0 to 2.8 x 10^7 cell ml^{-1}, which proved that the number of Cmm cells is similar in all samples, irrespective of the label used or treatment given. The total counts and FCM counts of non-heat treated cells labeled with Calcein AM (Fig. 3) or cFDA (Fig. 4) were almost the same, but the number of CFU ml^{-1} detected by plate counts was much lower. A linear relation ($r^2 \geq 0.95$) was found between the percentage of non-treated cells present in the samples and the FCM counts for Cmm cells labeled with Calcein AM or cFDA.

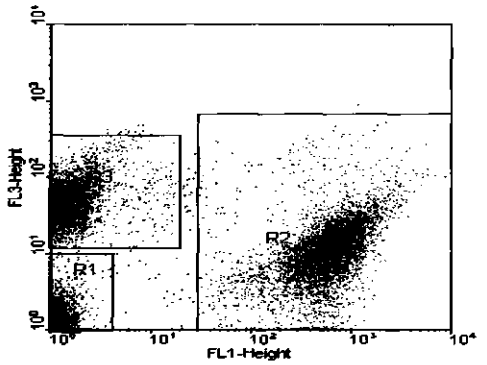

Fig. 2. Green fluorescence intensity (FL1) and red fluorescence intensity (FL3) of a Cmm population containing 50% heat-treated cells labeled with Calcein AM (R2) and PI (R3). R1 = background.

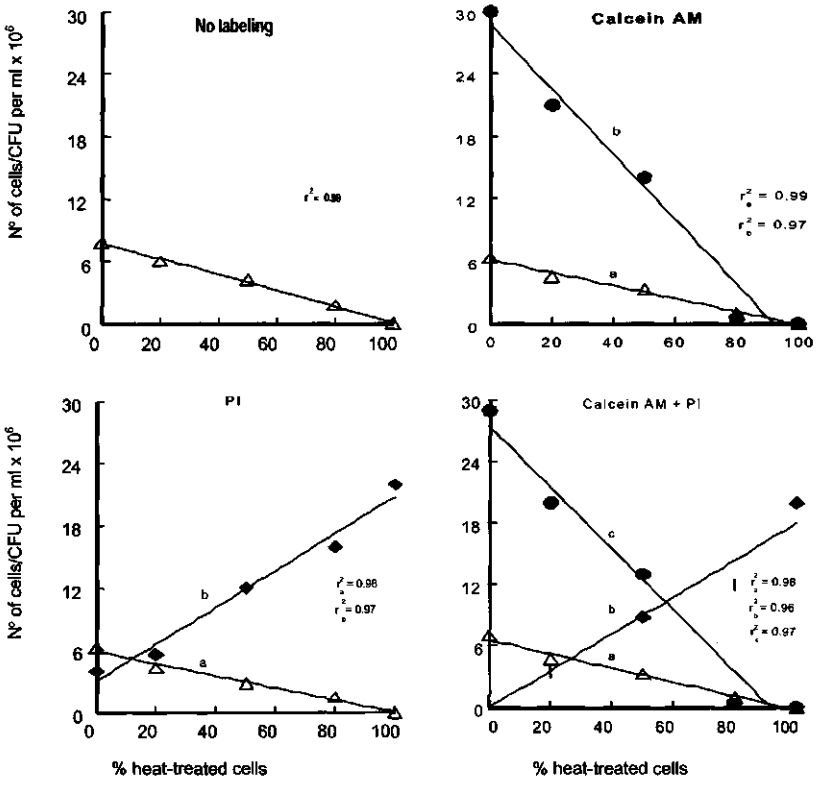

Fig. 3. Comparison between plate counts (Δ) and flow counts for different ratios of non-treated and heat-treated Cmm cells labeled with Calcein AM (●) or PI (◆).

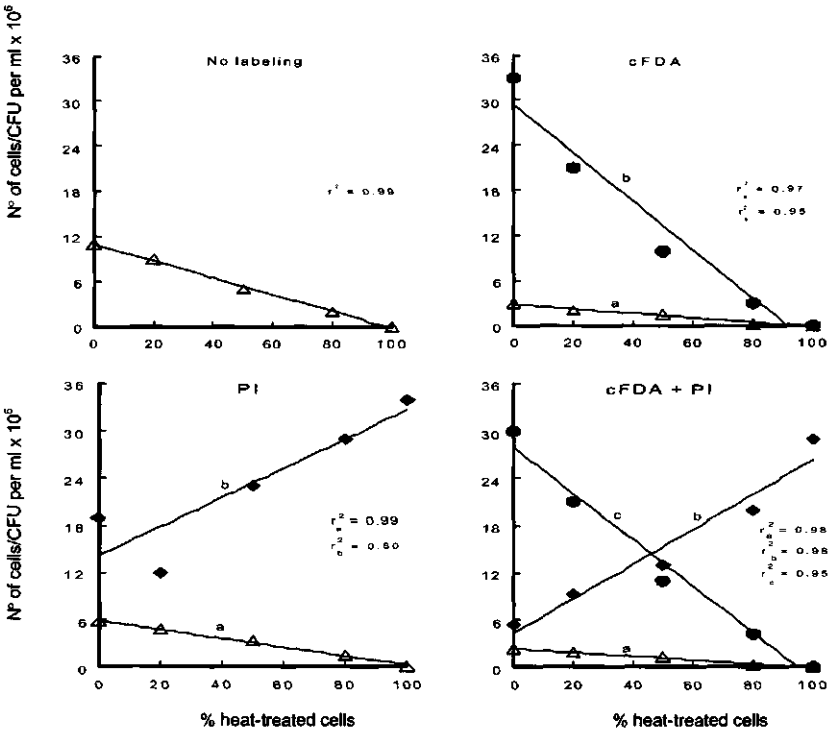

Fig.4. Comparison between plate counts (Δ) and flow counts for different ratios of non-treated and heat-treated Cmm cells labeled with cFDA (●) or PI (◆).

A linear relation ($r^2 \geq 0.80$) was also found between the percentage of heat-treated cells in the samples and the FCM counts for Cmm cells labeled with PI. However, the labeling with cFDA and PI appears to affect outgrowth of the Cmm cells after plating, since the number of CFU ml^{-1} showed a decrease of 72% and 52% compared to non-labeled cells, respectively. The plate counts showed that the recovery of Cmm cells in the presence of Calcein AM was higher than in the presence of cFDA. Indeed, the sorting of cells labeled with cFDA showed that only 0.6% to 5% of these cells could form colonies after plating (Table 1). For Cmm cells labeled with Calcein AM , 42 to 65% of the sorted cells were able to form colonies. As expected, sorted cells labeled with PI could not be recovered at all after plating on GNA.

Overall, the number of non-treated Cmm cells labeled with Calcein AM, cFDA, PI, or a combination of these probes, quantified by FCM analyses was comparable to the number of total counts and both were higher than the number of CFU ml^{-1} detected by plate counting.

Table 1. Colony formation of sorted Cmm cells labeled with Calcein AM, cFDA, PI or combinations of Calcein AM and cFDA with PI, after plating on GNA medium.

Label	Cmm population % heat treated cells	Concentration of sorted cells (cells/ml)	Plate counts of sorted cells CFU/ml)
Calcein AM	0	2.4×10^3	9.8×10^2 (42%)
PI	100	4.8×10^2	0
Calcein AM + PI	50	9.7×10^2 (Calcein AM stained) 4.2×10^2 (PI stained)	6.3×10^2 (65%) 0
cFDA	0	3.9×10^3	2.0×10^2 (5%)
PI	100	2.6×10^3	0
cFDA + PI	50	2.5×10^3 (cFDA stained) 3.5×10^3 (PI stained)	1.6×10 (0.6%) 0

Discussion

The assessment of the viability of the plant pathogenic bacterium Cmm applying the fluorescent probes Calcein AM, cFDA and PI in combination with flow cytometry was evaluated for Cmm populations differing in the ratio of viable cells. The use of flow cytometry to distinguish between viable and non-viable bacteria after labeling with cFDA, Calcein AM or PI has been reported before for microorganisms in food, compost extracts and seawater (Diaper and Edwards 1994a, 1994b; Magariños *et al.* 1997). The viability of Cmm cells in suspension has also been determined by measuring the intracellular pH as a viability indicator, using the pH dependent fluorescent probe 5 (and 6-)-carboxyfluorescein succinimidyl ester (cFSE) and FCM (Chitarra *et al.* 2000). However, compared to the method applied in this study, the intracellular pH analysis has to be performed at two pH values, the preparation of the samples takes a longer time, a calibration curve has to be made each time, and the results are more difficult to interpret.

In this study, we observed that non-treated (viable) and heat-treated (non-viable) Cmm cells could be distinguished based on the fluorescence intensity of the cells after labeling with Calcein AM or cFDA (green fluorescence), and PI (red fluorescence). A good correlation was found between the percentage of viable Cmm cells and the FCM counts when the cells were Calcein AM-stained. These results differ from the results reported by Kaneshiro *et al.* (1993) and Diaper and Edwards

(1994b). Their research showed the inability of Calcein AM to stain many yeast and bacterial cells, probably due to a poor accessibility of the cells for this dye.

cFDA is one of the mostly used fluorogenic esters that has been applied to detect viable bacteria using fluorescence microscopy (Chrzanowski *et al.* 1984) and flow cytometry (Diaper and Edwards 1994a, 1994b). Since this dye preferentially stains gram positive bacteria, it was expected to be a good viability indicator for Cmm. However, comparing cFDA and Calcein AM, the latter showed more reliable results, and appears to be a good indicator for viability of Cmm cells when applied in combination with FCM.

PI is a dye that is supposed not to cross intact cell membranes (Alvarado-Aleman *et al.* 1996), but it was able to stain 18 to 56% of non-treated Cmm cells when applied as a single stain. These results suggest that PI cannot be considered a good viability indicator for viable Cmm cells when it is applied alone. However, it was shown that it can be a good indicator for dead or demaged cells.

The number of labeled cells detected by FCM analyses was always higher than the number of CFU ml^{-1} detected by the plate count method. This implies that a large proportion of the cells are enzymatically active, e.g. they are able to hydrolyse Calcein AM or cFDA, but only about 55% and 24% are able to form colonies, respectively. Nonetheless, both plate count and FCM results show a linear relationship with the percentage of viable Cmm cells in the population. FCM counts can therefore be used as a viability indicator as well.

Overall, FCM analysis of Calcein AM stained cells was shown to be a method to assess viability of Cmm cells in suspensions with the speed of the assay being superior to the conventional plate count method.

Acknowledgement

We thank the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES (Brasil) for providing financial support to L. G. Chitarra during this study.

References

Allman, R., Hann, A.C., Manchee, R. and Lloyd, D. (1992) Characterization of bacteria by multiparameter flow cytometry. *Journal of Applied Bacteriology* **73:** 438-444.

Alvarado-Aleman, F.J., Collazo-Ponce, A. and Kumate-Rodrigues, J. (1996) Assessment of viability of yeast by flow cytometry using light green as vital dye exclusion. *Rev. Lat.-Amer. Microbiol.* **38:** 25-30.

Bernander, R., Stokke, T. and Boye, E. (1998) Flow cytometry of bacterial cells: comparison between different flow cytometers and different DNA stains. *Cytometry* **31:** 29-36.

Breeuwer, P., Drocourt, J.L., Rombouts, F.M. and Abee, T. (1996) A novel method for continuous determination of the intracellular pH in bacteria with the internally conjugated fluorescent probe 5 (and 6-)-carboxyfluorescein succinimidyl ester. *Applied and Environmental Microbiology* **62:** 178-183.

Bunthof, C.J., van den Braak, S., Breeuwer, P., Rombouts, F.M. and Abee, T. (1999) Rapid fluorescence assessment of the viability of stressed *Lactococcus latis*. *Applied and Environmental Microbiology* **65:** 3681-3689.

Chitarra, L.G., Breeuwer, P., van den Bulk, R.W. and Abee, T. (2000) Rapid fluorescence assessment of intracellular pH as a viability indicator of *Clavibacter michiganensis* subsp. *michiganensis*. *Journal of Applied Microbiology* **88:** 809-816.

Christensen, H., Bakken, L.R. and Olsen, R.A. (1993) Soil bacterial DNA and biovolume profiles measured by flow-cytometry. *FEMS Microbiology Ecology* **102:** 129-140.

Chrzanowski, T.H., Crotty, R.D., Hubbard, J.G. and Welch, R.P. (1984) Applicability of the fluorescein method of detecting active bacteria in freshwater. *Microbial Ecology* **10:** 179-185.

Crissman, H.A., Orlicky, D.J. and Kissane, R.J. (1979) Fluorescent DNA probes for flow cytometry. Considerations and prospects. *J. Histochem. Cytochem.* **27:** 1654-1655.

Davis, M.J., Gillaspie, A.G.Jr., Vidaver, A.K. and Harris, R.W. (1984) *Clavibacter*: a new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *xyli* sp. nov., subsp. nov. & *Clavibacter xyli* subsp.

cynodontis subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and bermudagrass stunting disease. *International Journal of Systematic Bacteriology* **34:** 107-117.

Diaper, J.P. and Edwards, C. (1994a) Flow cytometric detection of viable bacteria in compost. *FEMS Microbiology Ecology* **14:** 213-220.

Diaper, J.P. and Edwards, C. (1994b) The use of fluorogenic esters to detect viable bacteria by flow cytometry. *Journal of Applied Bacteriology* **77:** 221-228.

Edwards, C., Diaper, J.P., Porter, J. and Pickup, R. (1993) Applications of flow cytometry in bacterial ecology. In: Lloyd, D. (ed.). *Flow cytometry in microbiology*. London, UK: Springer-Verlag. pp. 121-129.

Endo, H., Nakayama, J., Hayashi, T. and Watanabe, E. (1997) Application of flow cytometry for rapid determination of cell number of viable bacteria. *Fisheries Science* **63:** 1024-1029.

Fry, J.C. (1990) Direct methods and biomass estimation. *Methods in Microbiology* **22**: 41-85.

Haugland, R.P. (1996) Handbook of fluorescent probes and research chemicals, 6th ed. *Molecular Probes*, Inc., Eugene, Oregon, USA, 679 pp.

Hudson, B., Upholt, W.B., Divinny, J. and Vinograd, J. (1969) The use of an ethidium bromide analogue in the dye-buoyant density procedure for the isolation of closed circular DNA: the variation of the superhelix density of mitocondrial DNA. *Proc. Natl. Acad. Sci.* USA. **62:** 813-820.

Kaneshiro, E.S., Wyder, M.A., Wu, Y.P. and Cushion, M.T. (1993) Reliability of calcein acetoxy methyl ester and ethidium homodimer or propidium iodide for viability assessment of microbes. *Journal of Microbiological Methods* **17:** 1-16.

Kaprelyantes, A.S. and Kell, D.B. (1992) Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry. *Journal of Applied Bacteriology* **72:** 410-422.

Kaprelyantes, A.S. and Kell, D.B. (1993a) The use of 5-cyano-2,3-ditolyl tetrazolium chloride and flow cytometry for the visualization of respiratory activity in individual cells of *Micrococcus luteus*. *Journal of Microbiological Methods* **17:** 115-122.

Kaprelyantes, A.S. and Kell, D.B. (1993b) Dormancy in stationary phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. *Applied and Environmental Microbiology* **59:** 3187-3196.

Langsrud, S. and Sundheim, G. (1996) Flow cytometry for rapid assessment of viability after exposure to a quartenary ammonium compound. *Journal of Applied Bacteriology* **81:** 411-418.

Magariños, B., Romalde, J.L., Cid, A. and Toranzo, A.E. (1997) Viability of starved *Pasteurella piscicida* in seawater monitored by flow cytometry and the effect of antibiotics on its resuscitation. *Letters in Applied Microbiology* **24:** 122-126.

Mason, D.J., Lopes-Amoros, R., Allman, R., Stark, J.M. and Lloyd, D. (1995) The ability of membrane potencial dyes and calcafluor white to distinguish between viable and non-viable bacteria. *Journal of Applied Bacteriology* **78:** 309-315.

Miles, A.A. and Misra, S.S. (1933) The estimation of the bacterial power of the blood. *The Journal of Hygiene* **38:** 732-749.

Plihon, F., Taillandier, P. and Strehaiano, P. (1995) A direct and simple method for rapid counting viable chains of *Leuconostoc* in batch cultures. *Biotechnology Techniques* **9:** 451-456.

Porter, J., Pickup, R. and Edwards, C. (1997) Evaluation of flow cytometric methods for the detection and viability assessment of bacteria from soil. *Soil Biol. Biochem.* **29:** 91-100.

Roth, B.L., Poot, M., Yue, S.T. and Millard, P.J. (1997) Bacterial viability and antibiotic susceptibility testing with sytox green nucleic acid stain. *Applied and Environmental Microbiology* **63:** 2421-2431.

Sgorbati, S., Barbesti, S., Citterio, S., Besbetti, G. and De Vecchi, R. (1996) Characterization of number, DNA content, viability and cell size of bacteria from natural environments using DAPI/PI dual staining and flow cytometry. *Minerva Biotecnologica* **8:** 9-15.

Taylor, I.W. and Milthorpe, B.K. (1980) An evaluation of DNA fluorochromes, staining techniques, and analysis for flow cytometry. I. Unperturbed cell population. *J. Histochem. Cytochem.* **28:** 1224-1232.

Ueckert, J.E., Von-Caron, G.N., Bos, A.P. and Steeg, P.F. (1997) Flow cytometric analysis of *Lactobacillus plantarum* to monitor lag times, cell division and injury. *Letters in Applied Microbiology* **25:** 295-299.

Widholm, J.M. (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Technol.* **47:** 189-194.

Williams. S.C., Hong, Y., Danavall, D.C.A., Howard-Jones, Gibson, M.H.D., Frischer, M.E. and Verity, P.G. (1998) Distinguish between living and nonliving

bacteria: evaluation of the vital stain propidium iodide and its combined use with molecular probes in aquatic samples. *Journal of Microbiological Methods* **32:** 225-236.

Chapter 4

Detection of the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* applying fluorescent antibodies and flow cytometry

L.G. Chitarra,[1,2] J.H.W. Bergervoet,[1] T. Abee[2] and R.W. van den Bulk[1]

[1] Department of Plant Development and Reproduction, Plant Research International B.V., Wageningen, The Netherlands

[2] Department of Food Technology and Nutritional Sciences, Wageningen University, Wageningen, The Netherlands

Summary

Xanthomonas campestris pv. *campestris* (Xcc) is a seed transmitted plant pathogenic bacterium which causes black rot of crucifers. Seed lots and plants are screened for contamination with this pathogen using plating or serological assays. These methods, however, are time consuming and not very sensitive, respectively. Therefore, flow cytometry (FCM) was evaluated for the rapid detection of Xcc cells labeled with a FITC-monoclonal antibody in pure culture and in mixed culture with the common saprophyte *Pseudomonas fluorescens* (Psf). The detection limit employing FCM was assessed and compared with the conventional plate count technique.

The monoclonal antibody (Mab) 18G12, directly conjugated with FITC, was used at dilutions of 1:50, 1:100, 1:200 and 1:400. For mixed suspensions of Xcc and Psf, Mab 18G12 was used at a dilution of 1:100. The analyses were performed with a Coulter EPICS XL-MCL flow cytometer, at low flow rate during 4 minutes. Serial ten-fold dilutions were made from the bacterial suspensions and plated in triplicate on sectored Tryptone Soya Agar medium.

Using FCM, Xcc cells labeled with FITC-conjugated monoclonal antibodies could rapidly be detected at low numbers, i.e 10^3 colony forming units ml^{-1} in pure and mixed cultures with Psf.

FCM in combination with Xcc specific FITC-labeled monoclonal antibodies may provide a novel tool for rapid detection of this plant pathogenic bacterium.

Introduction

Xanthomonas campestris pv. *campestris* (Xcc) is a seed-transmitted plant pathogenic bacterium which causes black rot of crucifers. The bacterium attacks *Brassica* spp, radishes and numerous weeds (Williams 1980). To prevent black rot, disease-free seeds should be used.

Several detection methods for Xcc have been developed to test seed lots and plants for contamination with the pathogen. The routinely used methods are based on plate assays or on serological tests. Plate assays allow isolation of the pathogen by plating seeds or seed extracts on semi-selective agar media (Schaad and Donaldson 1980; Randhawa and Schaad 1984; Chang *et al.* 1990; Chang *et al.* 1991). Semi-selective media may reduce the interference of saprophytes, but it can also affect the recovery of the target bacterium (Chun and Alvarez 1983). Saprophytic bacteria may produce antibiotics that can act in combination with inhibiting components in the selective media suppressing growth of Xcc (Schaad *et al.* 1997). Furthermore, plating assays require long incubation times, varying from 3 to 5 days.

Serological tests, such as the enzyme-linked immunosorbent assay (ELISA), register the occurrence of antigen-antibody complexes by rapid enzymatic development of a distinctly coloured product. Xcc could be detected on infected cabbage leaves using polyclonal antibodies within 5 hours, but the detection limit was quite high, i.e. 10^5 CFU ml^{-1} (Alvarez and Lou 1985). This detection method, therefore, is generally considered to be insensitive.

Alternatively, immunofluorescence microscopy (IF) has been evaluated for detecting FITC-labeled Xcc cells (Franken 1992). The reliability of this method, as well as ELISA, depends on the specificity of the antibodies, which must be tested prior to use and preferably should not cross-react with other bacteria present in the sample. The IF method requires at least 5 hours to prepare the samples. Furthermore, the examination of IF slides is time-consuming and should be performed by

experienced technicians. Immunofluorescence colony-staining (IFC) is based on a combination of plating and serological techniques. The specificity of IFC relies on growth on a selective medium, which can take 1 to 2 days, colony phenotype, and serological staining characteristics (Van Vuurde *et al.* 1995). Overall, the above mentioned methods are time consuming or not very sensitive.

Flow cytometry (FCM) is a technique that allows rapid examination and detection of individual cells based on their physical and/or chemical characteristics such as size, granularity or DNA content. FCM in combination with fluorescent antibodies has successfully been used to detect *Staphylococcus aureus* in lake water (Diaper *et al.* 1992), to detect *Escherichia coli* in natural lake water populations and sewage (Porter *et al.* 1993), to detect low levels of *Salmonella* in pure cultures (McClelland and Pinder 1994a; Pinder and McClelland 1994), and in dairy products (McClelland and Pinder 1994b). Advantages of FCM are its speed, the actual analysis can be done in a matter of minutes, and the fact that it is a quantitative method.

In this study, FCM was evaluated for the detection of Xcc cells labeled with a FITC-labeled monoclonal antibody in pure and in mixed cultures with the commonly occurring saprophytic bacterium *Pseudomonas fluorescens* (Psf).

Materials and Methods

Growth conditions

Xanthomonas campestris pv. *campestris* (Xcc) 1279A (HRI - Horticulture Research International, England) *and Pseudomonas fluorescens* (Psf) 252 (PRI - Plant Research International, The Netherlands) were grown on 1% Glucose-Nutrient-Agar (GNA; Oxoid) medium for 24 hours at 25°C. The cells were harvested and resuspended in 0.2M phosphate buffer (PB), pH 7.2. The Optical Density ($O.D_{620}$) was measured with a spectrophotometer at 620 nm and adjusted by diluting with PB to approximately 0.35 and 0.43 for Xcc and Psf respectively, in order to obtain suspensions with 10^7 to 10^8 cells ml^{-1}.

Bacterial sample preparation

For flow cytometric analyses, serial dilutions of the Xcc suspension were made in PB pH 7.2 to give cell concentrations ranging from approximately 10^2 to 10^6 cells ml^{-1}.

Suspensions of Xcc and Psf, each containing approximately 10^6 cells ml^{-1}, were mixed in different ratios, i.e. 100/0, 80/20, 50/50, 20/80, and 0/100%, prior to staining in order to test the monoclonal antibody specificity.

Cell labeling with monoclonal antibody

The monoclonal antibody (Mab) 18G12, directly conjugated with fluorescein isothiocyanate (FITC), was provided by IPO (Research Institute for Plant Protection, Wageningen, The Netherlands), and is specific for Xcc (Franken 1992). For FCM experiments, Mab 18G12 was tested at dilutions of 1:50, 1:100, 1:200 and 1:400. For mixed suspensions of Xcc and Psf, Mab 18G12 was used at a dilution of 1:100. The samples were stained by incubation with Mab at room temperature for 30 minutes in the dark. Subsequently, the cells were spun down at 11000 g, washed, resuspended in PB pH 7.2 and placed on ice until required.

Flow cytometry

All the analyses were performed with a Coulter EPICS XL-MCL flow cytometer (Beckman-Coulter Electronics, Miami, FL, USA) equipped with a 15 mW Argon ion laser at 488 nm. Green fluorescence emission of the monoclonal antibody 18G12-FITC conjugate was measured with a band pass filter at 525 nm (510-540 nm). Forward scatter was used as an indicator of cell size and the side scatter was used as an indicator of cell granularity. The flow rate of the system was calibrated using Flow Count (Beckman-Coulter Electronics, Miami, FL, USA) with a diameter of 10 μm, at a concentration of 997 particles μl^{-1}. The calibration was performed in triplicate for 4 minutes at a low flow rate. Subsequently, the volume ($\mu l\ min^{-1}$) of each analysed sample was calculated. An average volume of 9.5 $\mu l\ min^{-1}$ was used as a standard value to calculate the number of FITC-labeled Xcc cells present in each sample in further experiments.

The sample analysis time was 4 minutes, and the cells were separated from background by their side and forward scatter characteristics. The number of labeled-FITC cells present in each sample was calculated based on the peak of the histogram (number of FITC-labeled Xcc cells), time of analysis (4 minutes), and volume (38 μl). The data are presented as the total amount of FITC-labeled cells ml^{-1} in each sample.

Plate count technique

Plate counts were determined as described by Miles and Misra (1933). Basically, serial dilutions were made from both stained and unstained bacterial suspensions in PB pH 7.2, and 20 µl drops of each dilution were plated in triplicate on sectored Tryptone Soya Agar (TSA, Oxoid) plates. After incubation of plates for 48 hours at 25°C, the number of colonies was counted for those dilutions producing between 3 and 30 colonies per 20 µl drop, and the total number of colony-forming units ml^{-1} (CFU ml^{-1}) was calculated.

Total cells counts

Four replicates of unstained and stained 100-fold diluted samples were counted in a Neubauer counting chamber with a fluorescence microscope (Zeiss, Axiophot, West Germany). The total count determination was done by counting labeled Xcc cells to enable discrimination between Xcc and Psf.

Results

Detection level of FITC-labeled Xcc cells

To establish the potential use of flow cytometry for the detection of the plant pathogenic bacterium Xcc, the optimal concentration of the Mab and the detection limit of the flow cytometry method were determined.

Ten-fold serial dilutions of Xcc, ranging from 10^2 to 10^6 CFU ml^{-1} as determined by plate counts, were used for this purpose. Detection by flow cytometry of Xcc cells labeled with FITC-monoclonal antibody gave reliable counts for suspensions of 10^3 CFU ml^{-1} or higher (Table 1), indicating that 10^3 CFU ml^{-1} is the detection limit for flow cytometry detection of Mab-FITC-labeled Xcc.

Table 1. Comparison of plate counts and flow counts for serial dilution of FITC-labeled Xcc cells. Cells were labeled with Mab 18G12 at dilution of 1:100, and the initial O.D $_{620}$ of the undiluted sample was 0.35.

Xcc dilutions	Plate counts (cfu ml^{-1})	Flow counts (cells ml^{-1})
-3	5.7×10^5	6.1×10^5
-4	5.7×10^4	6.8×10^4
-5	1.0×10^4	9.4×10^3
-6	1.2×10^3	2.3×10^3
-7	0	1.0×10^3
-8	0	1.9×10^3

For the series of different Mab dilutions, good correlation was observed between the flow cytometry counts and the plate counts for bacterial suspensions containing 10^3 to 10^6 CFU ml^{-1} (Fig. 1).

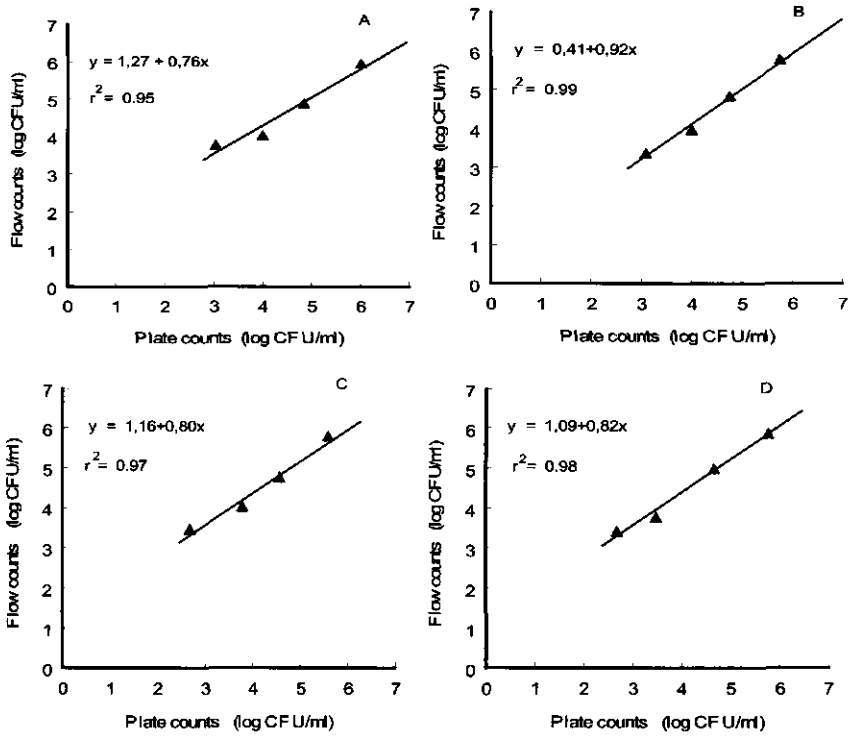

Fig. 1. Correlation between plate counts (CFU ml^{-1}) and flow counts (cells ml^{-1}) for 10-fold serial dilution of Xcc and different working titers for Mab 18G12: A = 1:50; B = 1:100; C = 1:200, and D = 1:400.

These results also show that the concentration of the Mab is an important factor for optimal detection of Xcc using FCM. Among the Mab dilutions, the dilutions of 1:50 and 1:100 showed relatively less background compared to the dilutions of 1:200 and 1:400, but with the dilution 1:50 a low correlation between plate counts and flow counts was observed. Of all events (including background and cell counts), 80% of the counts detected by FCM represented FITC labeled Xcc cells for Mab dilutions of 1:50 and 1:100, whereas 69% and 55% of all events were labeled Xcc cells for Mab dilutions of 1:200 and 1:400, respectively (Fig. 2). It was also

observed in previous experiments that FITC-labeled Xcc cells with Mab 18G12 always gave less background at dilutions of 1:50 and 1:100 (data not shown). Therefore, the Mab dilution of 1:100 was chosen for further experiments because the background was low and it gave the best correlation between plate counts and flow counts.

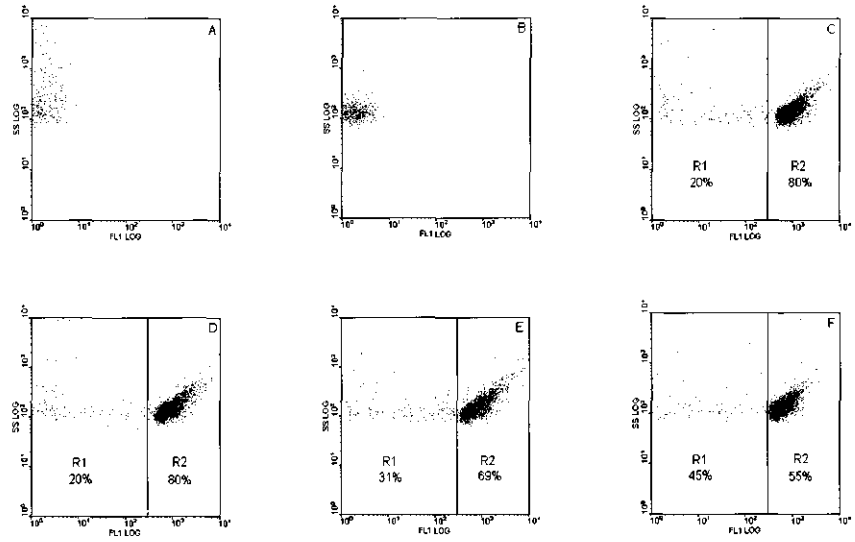

Fig. 2. Fluorescence intensity (FL1) of FITC-labeled Xcc cells (R2) and the background (R1) for different working titers of Mab 18G12; C = 1:50; D = 1:100; E = 1:200 and F = 1:400. A and B represent background and non-labeled Xcc cells, respectively.

The total counts always showed higher numbers of cells than flow counts and plate counts (Table 2).

Table 2. Comparison of total counts, flow counts, and plate counts of FITC-labeled Xcc cells at different working titers of Mab 18G12.

Mab18G12 Working titer	Total counts cells ml^{-1}	Flow counts cells ml^{-1}	Plate counts CFU ml^{-1}
1:50	2.9×10^6	9.1×10^5	1.0×10^6
1:100	2.0×10^6	6.1×10^5	5.7×10^5
1:200	1.7×10^6	6.2×10^5	3.8×10^5
1:400	2.5×10^6	7.3×10^5	5.8×10^5

Detection of Xcc in the presence of *P. fluorescens*

Both plate assays and flow cytometry allowed detection of Xcc cells labeled with the Mab-FITC conjugate when mixed with the saprophyte Psf in different ratios. The amount of labeled Xcc cells detected was proportional to the percentage of cells present in the samples. Labeled Xcc cells showed relatively high green fluorescence levels (FL1) compared to non-labeled cells and the saprophyte Psf (Fig. 3).

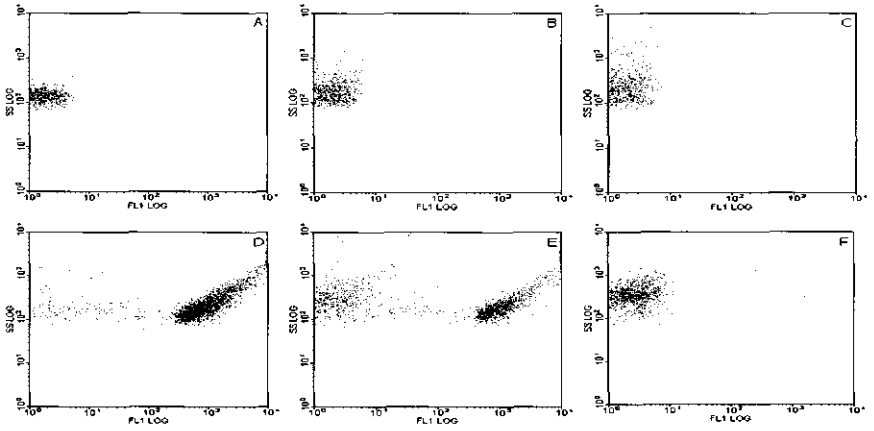

Fig. 3. Fluorescence intensity (FL1) of Xcc and Psf cells without (A,B, and C) and with Mab 18G12 (D,E, and F). A and D = 100% Xcc; B and E = 50% : 50% Xcc and Psf cells; C and F = 100% Psf cells.

The highest numbers of labeled Xcc cells were obtained using the total count technique (Fig. 4), whereas plate counts gave the lowest counts.

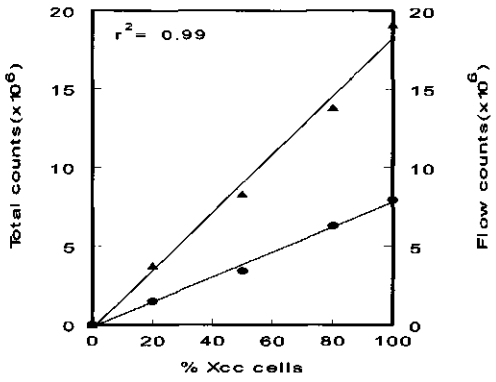

Fig. 4. Correlation between the number of Xcc cells labeled with Mab 18G12, in the presence of an increased percentage of Psf cells, determined by the total count technique (▲) and flow cytometry (●).

A good correlation (r^2 = 0.95) was observed between the flow cytometry counts and plate counts, although flow counts were always higher than plate counts (Fig. 5).

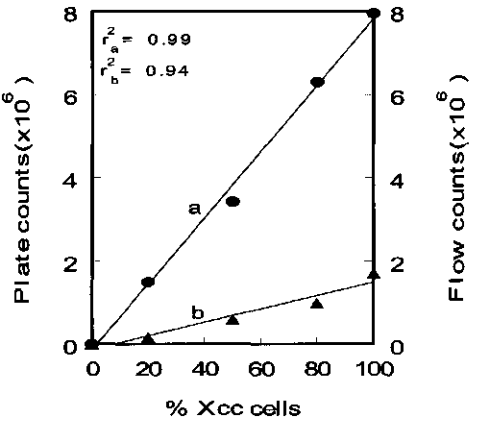

Fig. 5. Correlation between the number of Xcc cells labeled with Mab 18G12 at dilution of 1:100, in the presence of an increased percentage of Psf cells, determined by the plate count technique (▲) and flow cytometry (●).

Discussion

This is the first report which shows that FCM in combination with Xcc specific FITC-labeled monoclonal antibodies allows rapid detection of a plant pathogen bacterium. Including the incubation time for staining, the analysis could be performed within one hour. Combined with the Multi Carousel Loader (MCL), this can speed up test procedures considerably.

It was shown in this study that the concentration of Mab affected the sensitivity of the FCM measurements. This has also been reported for the sensitivity of serological techniques such as IF and IFC (Franken 1992; Van Vuurde 1997). This is based on the concept that the optimal concentration of Mab is the one that gives the greatest discrimination between the positive cells (fluorescence staining-signal) and negative cells (non-specific binding). FCM measurements were less sensitive and showed higher backgrounds as the concentration of Mab decreased (Fig. 2). Though, it seems not to be a limiting factor for the detection of Xcc by flow cytometry. In our

study, the limitations of the flow cytometry technique for the detection of Xcc cells labeled with FITC-antibody complex are primarily the concentration of the cells present in the samples and the fact that Mab does not discriminate between live and dead cells. The detection limit for Xcc using the FCM method is 10^3 CFU ml^{-1}. The sensitivity of flow cytometry for the detection of *Salmonella* spp. in pure cultures, or in a food matrix, proved to be reliable down to the number of 1 x 10^4 cells ml^{-1} (McClelland and Pinder 1994a, 1994b).

The detection limit for plant pathogens in plant materials and environmental samples applying the conventional plating technique and the IFC method is limited to about 10^4 and 10^2 CFU ml^{-1}, respectively (Van Vuurde *et al* 1995). For Xcc, the detection threshold is important, since the tolerance level for black rot of crucifers is rather low. Only one infected cabbage seed per 10.000 seeds is acceptable for direct seeding of cabbage (Schaad *et al.* 1980). Seed health tests which are applied to test seed lots for contamination with pathogens are usually based on the conventional plate assays. Such assays take a long time and meanwhile, seed industries have to store the seeds before they can be processed and become available to the market. This results in a significant increase in cost. A reliable, fast and sensitive method to detect Xcc is therefore highly desirable. As shown, FCM combined with monoclonal antibodies may provide an alternative detection method for Xcc cells. The only prerequisite for the use of antibodies is that no cross-reactions with other bacteria present in the sample occur. In this study, no cross-reactions were observed with the common saprophyte Psf, independent of the ratio Xcc/Psf tested. The monoclonal antibody used in this study was previously shown to be specific for Xcc, although a mixture of 3 different Mabs was recommended for use in routine seed health testing for Xcc (Franken 1992). One of the advantages of applying the FCM technique for the detection of Xcc is the short assay time, i.e. less than 1 hour. For these reasons, FCM combined with Xcc specific FITC-monoclonal antibodies may provide a novel tool for rapid detection of this plant pathogenic bacterium.

Acknowledgement

We thank the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES (Brasil) for providing financial support to L. G. Chitarra during this study.

References

Alvarez, A.M. and Lou, K. (1985) Rapid identification of *Xanthomonas campestris* pv. *campestris* by ELISA. *Plant Disease* **69:** 1082-1086.

Chang, C.J., Donaldson, D., Crowley, M. and Pinnow, D. (1991) A new semiselective medium for the isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds. *Phytopathology* **81:** 449-453.

Chang, C.J., Donaldson, R., Crowley, R. and Pinnow, D. (1990) Isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds using a highly selective medium. In: Klement, Z. (ed.). *Proceedings of the 7th International Conference on Plant Pathogenic Bacteria.* Hungary, Budapest: Akadémiai Kiadó. pp. 533-539.

Chun, W.W.C. and Alvarez, A.M. (1983) A starch-methionine medium for isolation of *Xanthomonas campestris* pv. *campestris* from plant debris in soil. *Plant Disease* **67:** 632-635.

Diaper J.P., Edwards, C., Stewart, T.D. and Sussman, M. (1992) The detection of bacteria in aquatic environments by flow cytometry. The release of genetically modified microorganisms: In: Stewart, T.D. (ed.). *Proceedings of a symposium held under the auspices of the Federation of European Microbiological Societies.* August 29-31, 1991, Nottingham, UK. pp. 233-234.

Franken, A.A.J.M. (1992) Application of polyclonal and monoclonal antibodies for the detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds using immunofluorescence microscopy. *Netherlands Journal of Plant Pathology* **98:** 95-106.

McClelland, R.G. and Pinder, A.C. (1994a) Detection of low levels of specific Salmonella species by fluorescent antibodies and flow cytometry. *Journal of Applied Bacteriology* **77:** 440-447.

McClelland, R.G. and Pinder, A.C. (1994b) Detection of *Salmonella typhimurium* in dairy products with flow cytometry and monoclonal antibodies. *Applied and Environmental Microbiology* **60(12):** 4255-4262.

Miles, A.A. and Misra, S.S. (1933) The estimation of the bacterial power of the blood. *The Journal of Hygiene* **38:** 732-749.

Porter, J., Edwards, C., Morgan, A.W. and Pickup, R.W. (1993) Rapid, automated separation of specific bacteria from lake water and sewage by flow cytometry and cell sorting. *Applied and Environmental Microbiology* **59(10):** 3327-3333.

Pinder, A.C. and McClelland, R.G. (1994) Rapid assay for pathogenic *Salmonella* organisms by immunofluorescence flow cytometry. *Journal of Microscopy* **176(1):** 17-22.

Randhawa, P.S. and Schaad, N.W. (1984) Selective isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds. *Phytopathology* **74:** 268-272.

Schaad, N.W. and Donaldson, R.C. (1980) Comparison of two methods for detection of *Xanthomonas campestris* in crucifer seeds. *Seed Science and Technology* **8:** 383-391.

Schaad, N.W., Sitterly, W.R. and Humaydan, H. (1980) Relationship of incidence of seedborne *Xanthomonas campestris* to black rot of crucifers. *Plant Disease* **64(1):** 91-92.

Schaad, N.W., Bonde, M.R. and Hatziloukas, E. (1997) BIO-PCR: a highly sensitive technique for detecting seedborne fungi and bacteria. In: Hutchins, J.D. and Reeves, J.C. (eds.). *Seed Health Testing Progress Towards the 21th Century.* Cambridge, UK. pp 159-164.

Van Vuurde, J.W.L., Kastelein, P. and Van der Wolf, J.M. (1995) Immunofluorescence colony-staining (IFC) as a concept for bacterial and ecological research. *EPPO Bulletin* **25:** 157-162.

Van Vuurde, J.W.L. (1997) Immunofluorescence colony-staining as a tool for sample indexing and for the determination of field thresholds for pathogenic seedborne bacteria In: Hutchins, J.D. and Reeves, J.C. (eds.). *Seed Health Testing Progress Towards the 21th Century*. Cambridge, UK. pp 165-173.

Williams, P.H. (1980) Black rot: a continuing thread to world crucifers. *Plant Disease* **64:** 736-742.

Chapter 5

Detection of the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* in seed extracts of *Brassica* sp. applying fluorescent antibodies and flow cytometry

L.G. Chitarra, C.J. Langerak, J.H.W. Bergervoet and R.W. van den Bulk
Plant Research International B.V., P.O. Box 16, 6700 AA, Wageningen, The Netherlands

Summary

The plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* (Xcc) is the causal organism of black rot of crucifers. The bacterium is seed-borne, and to prevent black rot, pathogen-free seeds must be used.

The routine methods used for the detection of Xcc in seed lots are usually based on plate assays and / or serological techniques. However, these methods are time consuming and laborious.

Flow cytometry (FCM) was evaluated as a tool for a rapid detection and quantification of Xcc cells labeled with a mixture of specific FITC-labeled monoclonal antibodies in crude seed extracts.

FCM allows a rapid detection and quantification of Xcc cells labeled with FITC-monoclonal antibodies (18G12, 2F4, and 20H6) in the samples tested. The presence of non-pathogenic Xc in the seed extracts did not interfere with FCM results. Xcc cells could be distinguished from cells of other organisms and small particles present in the seed extracts based on the high intensity fluorescence of labeled cells. FCM counts were always higher than plate counts which is most likely due to the fact that monoclonal antibodies do not discriminate between viable and non-viable cells.

The application of FCM in combination with FITC-monoclonal antibodies appears to be a promising technique for the detection and quantification of Xcc cells in seed extracts of crucifers.

Introduction

The plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* (Xcc) is a seed-borne bacterium which causes black rot of crucifers, one of the most important diseases of crucifers (Williams, 1980). To prevent black rot, the use of pathogen-free seeds is recommended. Testing of seed lots for the presence of the pathogen is, therefore, essential.

The routine methods used for the detection of Xcc in seed lots or in plants are usually based on plating assays and / or serological techniques. Plating assays are based on plating seed washings or extracts on selective or semi-selective media, and are considered to be reliable and efficient methods for routine detection (Schaad 1989). A major disadvantage of plating assays is the long incubation time required, taking for Xcc from 2 - 3 days up to one week. Another disadvantage is the possible presence of cells of other microorganisms, which may interfere by causing overgrowth or suppression of outgrowth of the target bacterium, in spite of the availability of semi-selective culture media.

Alternative methods which are available for detection of bacteria include immunological and DNA techniques. Both techniques can be performed in a shorter timespan than the plating assays. However, these techniques are considered to provide the user only with semi-quantitative information, which for seed health testing is not satisfactory. Furthermore, immunological techniques, such as enzyme-linked imunosorbent assay (ELISA), are relatively insensitive, and DNA-based methods are relatively expensive and more laborious.

Nowadays, advanced techniques have become available for simultaneous detection and quantification of specific plant pathogenic bacteria, even when they are present in low numbers. Flow cytometry (FCM) is such a technique, which combines the advantages of microscopy and serological analysis in a single, highly sensitive technique for a rapid examination and detection of numerous individual cells in a few minutes (Muirhead *et al.* 1985). Moreover, flow cytometry in combination with fluorescent probes technology has successfully been applied for a rapid and specific detection and enumeration of bacteria in medical, veterinary, and environmental microbiology (Pinder *et al.* 1990; Page and Burns 1991; Diaper *et al.* 1992; Li and Walker 1992; May *et al.* 1994; Porter *et al.* 1993; McClelland and Pinder 1994a, 1994b; Pinder and McClelland 1994; Kusunoki *et al.* 1998).

In this study, flow cytometry in combination with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies was evaluated as a new method for the detection and quantification of Xcc cells in pure culture, in suspensions containing both Xcc and Xc strains, and in crude seed extracts.

Materials and Methods

Seed lots

Healthy and naturally infected seed lots with three different levels of contamination were kindly provided by Bejo Zaden B.V., The Netherlands. The contamination level of the seed lots was based on plating results determined by Bejo Zaden B.V..

Organisms and growth conditions

The Xcc strains and Xc strains employed in this study are listed in table 1. All the strains were cultured on 1% Glucose-Nutrient-Agar (GNA; Oxoid) medium for 24 hours at 25°C. The cells were harvested and resuspended in sterile saline (0.85% NaCl). The optical density (O.D.) was measured with a spectrophotometer at 620 nm and adjusted by diluting with sterile saline to approximately 0.35, in order to obtain concentrations of approximately 10^6 colony-forming units per ml (CFU ml^{-1}).

Extraction of Xcc from cabbage seeds

Five sub-samples of 3,000 cabbage seeds of healthy seed lot were artificially contaminated in a 250 ml Erlenmeyer flask by adding 30 ml of pure suspensions of either Xcc 367, Xc 5040, or Xc 5053, or the mixture of Xcc 367 / Xc 5040 or Xcc 367 / Xc 5053. The sub-samples were shaken on an orbital shaker at 125 rpm for 5 minutes and then kept stationary at room temperature (22-25°C) for 2.5 hours. Subsequently, the extract of each sub-sample was filtered through a nylon filter with a mesh width of 10 µM, centrifuged for 3 minutes at 11,000*g*, washed, and resuspended in the same volume of 0.85% sterile saline.

Xcc was extracted from naturally contaminated sub-samples, each containing 3,000 seeds, by adding 30 ml of sterile saline, pre-chilled to 2 - 4°C. The extraction procedure for Xcc was performed as described above.

Isolation of Xcc from the seed extract

Ten-fold dilutions of each sub-sample were prepared in sterile saline. Fifty microliter aliquots of each dilution were plated in duplicate on NSCAA (Randhawa and Schaad 1984) and FS (Fieldhouse and Sasser unpublished; Schaad 1989) media. Inoculated NSCAA plates were incubated at 25°C for three days and FS plates for four days. The number of colonies was counted for those dilutions producing between 15 and 300 colonies per 50 µl, and the total number of CFUs ml^{-1} for each dilution was calculated. Reference strain Xcc 367 was plated as a control.

Cell labeling with monoclonal antibody

The mixture of monoclonal antibodies (Mabs) 18G12, 2F4, and 20H6, directly conjugated with fluorescein isothiocyanate (FITC), was provided by the former Research Institute for Plant Protection, Wageningen, The Netherlands, and was shown before to be specific for Xcc (Franken 1992). Pure suspensions of Xcc, mixed suspensions of Xcc and Xc, undiluted, and 10 and 100 times dilutions of the seed extract were incubated with the mixture of Mabs, at room temperature for 30 minutes in the dark, at a final titer of 1:100, before analysis by flow cytometry.

Flow cytometry

All the analyses were performed with a Coulter EPICS XL-MCL flow cytometer (Beckman-Coulter Electronics, Miami, FL, USA) equipped with a 15 mW Argon ion laser at 488 nm. Green fluorescence emission (FL1) of the mixture of FITC conjugated monoclonal antibodies was measured with a band pass filter at 525 nm (510-540 nm). Forward scatter was used as an indicator of cell size and the side scatter was used as an indicator of cell granularity. The flow rate of the system was calibrated using Flow Count (Beckman-Coulter Electronics, Miami, FL, USA) with a diameter of 10 µm, at a concentration of 997 particles per µl. The calibration was performed in triplicate for 4 minutes at a low flow rate. Subsequently, the volume (µl per minute) of each analysed sample was calculated. An average flow rate of 9.0 µl per minute was used as a standard value to calculate the number of FITC-labeled Xcc cells present in each sample in further experiments. The sample analysis time was 2 minutes, and the cells were separated from background on basis of their side and forward scatter characteristics. The number of FITC - labeled cells present in each

sample was calculated based on the peak of the histogram (FL1 - number of FITC-labeled Xcc cells), time of analysis (2 minutes), and volume (18 µl). The data are presented as the total amount of FITC-labeled cells ml^{-1} in each sample.

Immunofluorescence microscopy (IF)

The IF slides were prepared by pipetting 5 µl aliquots of each stained sample in duplicate on a Neubauer counting chamber. Reading of IF slides was done with a fluorescence microscope (Zeiss, Axiophot, West Germany), using a 40x objective magnification, 10x ocular magnification, and a I 2 filter system for incident illumination with blue light for FITC excitation. Xcc cells were considered IF positive when a high green fluorescence signal of FITC-labeled Xcc cells was observed. IF positive cells were counted.

Pathogenicity test

Xcc 367, Xcc 1279A, Xc 5040 and Xc 5053 were cultured on yeast extract-dextrose-calcium carbonate agar (YDC) (Schaad 1988) for 24 hours at 25°C. Two plants in the 3-4 leaf stage of each susceptible cabbage cultivar, Almanac and Erdeno (*Brassica oleracea* var. *capitata*), were inoculated. Two different methods of inoculation were used. In the first method, the major veins of the first two true leaves were stabbed at 5-10 points with a sterile toothpick contaminated with Xcc cells directly scraped from a culture on YDC medium. In the second method, the stem below the first two true leaves was carefully injected with a freshly prepared light milky Xcc suspension (10^7 - 10^8 cells ml^{-1}), using a hypodermic syringe. In both methods, tap water was used as a negative control. Plants were incubated in a growth cabinet at 20°C and 60% relative humidity, with a 12 hours light cycle. The appearance of typical V-shaped, yellow lesions with blackened veins after 7-10 days was considered to be a positive response.

Results

Specificity of the Mabs in flow cytometry

A good specificity of the monoclonal antibodies used in this study is essential for their application in either IF or FCM. To verify the possible occurrence of unspecific binding of the Mabs 20H6 (IgG1) and 18G12 (IgG3) and 2F4 (IgG3), isotypes of IgG1 and IgG3, FITC-conjugated, were tested. Pure suspensions of Xcc 367 and Xc

5040, and cabbage seed extracts containing mixed suspensions of Xcc 367 and Xc 5040 were used. No cells were added to the control samples. FCM analysis detected no green fluorescence signal from the FITC-labeled IgG1 and IgG3, showing that no unspecific binding occurred for all the samples tested, meaning that the Mabs applied in this study are specific for Xcc cells.

Pathogenicity testing

Xcc 367, 1279A and the *X. campestris* strains 5040 and 5053 were tested for pathogenicity in two susceptible cabbage cultivars, using two different methods of inoculation, stabbing with a toothpick and injecting with a syringe. Independent of the inoculation method used, Xcc 367 and Xcc 1279A produced on both cultivars systemic black discoloration of the veins after 7-10 days of inoculation, followed by appearance of leaf lesions and desiccation of the lesion tissue. *X. campestris* strains 5040, 5053, and the water control did not show any symptoms on the cultivars tested.

Table 1. Comparison of ELISA, flow cytometry, and immunofluorescence microscopy detection of Xanthomonas strains labeled with a mixture of Mabs (18G12, 2F4 and 20H6) FITC-conjugated. Xcc strains gave positive reaction and *X. campestris* strains negative reaction (non-pathogenic for cabbage).

Xanthomonas strains	Origin	*ELISA	FCM	IF
367	NCPPB 1645	n.d.	+	+
1279A	England	n.d.	+	+
5110	Australia	+	+	+
5112	France	+	+	+
5115	unknown	+	+	+
5117	unknown	+	+	+
5119	unknown	+	+	+
5120	unknown	+	+	+
5121	unknown	+	+	+
5128	Australia	+	+	+
5087	Italy	+	+	+
5005	unknown	-	-	-
5111	Italy	-	-	-
5113	unknown	-	-	-
5018	unknown	-	-	-
5020	unknown	-	-	-
5131	unknown	-	-	-
5037	Italy	-	-	-
5040	Italy	-	-	-
5041	Italy	-	-	-
5053	Italy	-	-	-
5064	Italy	-	-	-

n.d. not determined; + = positive reaction; - = negative reaction
*ELISA test was done by Bejo Zaden B.V., The Netherlands

Testing of various *X. campestris* strains by FCM and IF

Eleven Xcc strains and 11 other *X. campestris* strains were tested for their reaction with Mabs 18G12, 2F4, and 20H6 in IF and FCM. The test results were also compared with results from ELISA done with the same set of Mabs, obtained from Bejo Zaden B.V., The Netherlands (Table 1). All Xcc strains showed a positive response in either method, whereas cells of the (non-pathogenic) *X. campestris* were not detected.

Detection of Xcc by FCM in pure and mixed suspensions

FCM and IF were used to identify and to quantify Xcc cells labeled with the FITC-conjugated Mabs 18G12, 2F4 and 20H6 in pure suspensions and in suspensions containing a mixed population of Xcc 367 with Xc 5040 or Xc 5053. FCM analysis of pure suspensions of Xcc 367 showed a high intensity of the fluorescence signal (FL1) after labeling, whereas Xc strains 5040 and 5053 showed a low intensity of the fluorescence signal (FL1). In suspensions containing Xcc 367 and Xc 5040 or 5053, a high fluorescence signal was shown only by the labeled Xcc 367 cells (Fig. 1).

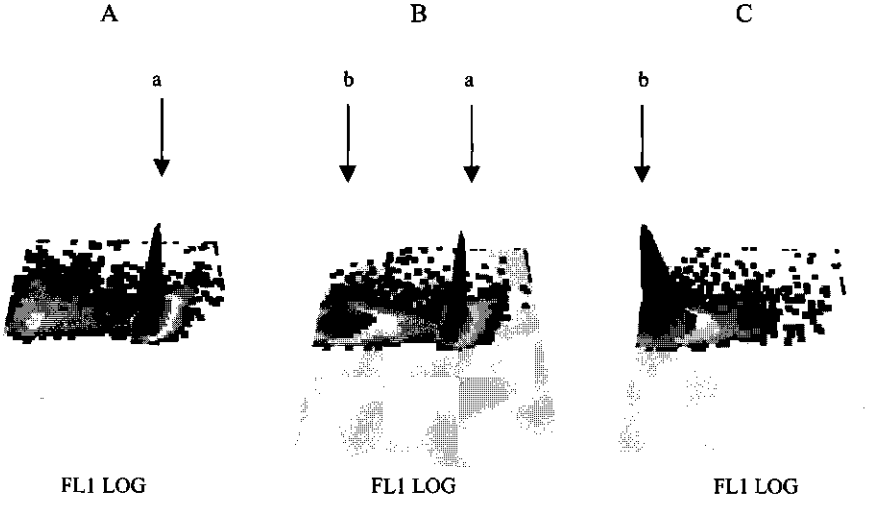

Fig. 1. Fluorescence intensity (FL1) of suspensions containing Xcc 367 (a) and / or 5053 (b) cells labeled with a mixture of FITC-conjugated monoclonal antibodies (18G12, 2F4, and 20H6). A = 100% Xcc 367; B = 50% : 50% Xcc 367 and P5053; C = 100% P5053.

FCM analysis of pure suspensions of Xcc 367 (Table 2), showed that 96.6% (3.4×10^6 cells ml^{-1}) of all events, background and cell counts, represented FITC-labeled Xcc 367 cells. For Xc 5040 and Xc 5053, FCM analysis showed that 0.38 (6.7×10^3 cells ml^{-1}) and 0.28% (6.3×10^3 cells ml^{-1}) of all events were labeled particles at the region were Xcc cells are suppose to be present, respectively.

Table 2. Detection by FCM, IF, and plate counts of Xanthomonas strains labeled with a mixture of FITC-conjugated Mabs (18G12, 2F4, and 20H6), at a dilution of 1:100, in pure suspensions and in seed extract. Bacterial cells were added to and extracted from cabbage seeds. The initial $O.D._{620} = 0.35$.

Xc strains	FCM (cells ml^{-1})	IF (cells ml^{-1})	Plate counts (CFU ml^{-1})
367	3.4×10^6	5.7×10^6	7.8×10^4
5040	6.7×10^3	0	6.8×10^4
5053	6.3×10^3	0	6.8×10^4
367 + 5040	1.5×10^6	3.7×10^6	8.2×10^4
367 + 5053	1.4×10^6	3.2×10^6	7.8×10^4
SE control	3.9×10^3	0	0
SE + 367	6.1×10^6	6.5×10^6	1.7×10^5
SE + 5040	5.2×10^3	0	1.5×10^5
SE + 5053	5.2×10^3	0	9.0×10^4
SE + 367 + 5040	2.6×10^6	4.7×10^6	9.6×10^4
SE + 367 + 5053	2.7×10^6	4.2×10^6	8.7×10^4

SE = seed extract

IF analysis showed that in mixed suspensions containing both Xcc 367 and Xc 5040 or Xc 5053, the green fluorescence observed was from FITC-labeled Xcc 367 cells, because Xc 5040 and Xc 5053 did not show fluorescence when analysed using IF microscopy.

All tested Xanthomonas strains were detected after plating the suspensions on FS and NSCAA media.

Detection of Xcc by FCM in artificially contaminated cabbage seed lots

Bacterial suspensions containing Xcc 367 or mixed suspensions of Xcc 367 with 5040 or 5053 were added to a healthy cabbage seed lot, which was then extracted and the extract incubated with a mixture of FITC-conjugated Mabs (18G12, 2F4 and 20H6) prior to FCM and IF analysis. FCM analysis of extracts containing Xcc 367 showed a high intensity of the fluorescence signal (FL1) and FCM could separate the target bacterium from the background, which contains non-labeled cells, other particles present in the samples, or non-specifically labeled particles (Fig. 2). Xc 5040 and

5053 showed a low intensity fluorescence signal, which was part of the background. IF analysis of the same extracts showed that only Xcc 367 cells were FITC-labeled. No fluorescent cells were observed in extracts containing pure suspensions of Xc 5040 or 5053. No Xcc cells were detected in the control seed extract samples as shown by IF and plate counts.

FCM and IF counts for all the samples tested were always higher than plate counts (Table 2), due to the fact that test methods applying Mabs do not discriminate between live and dead cells.

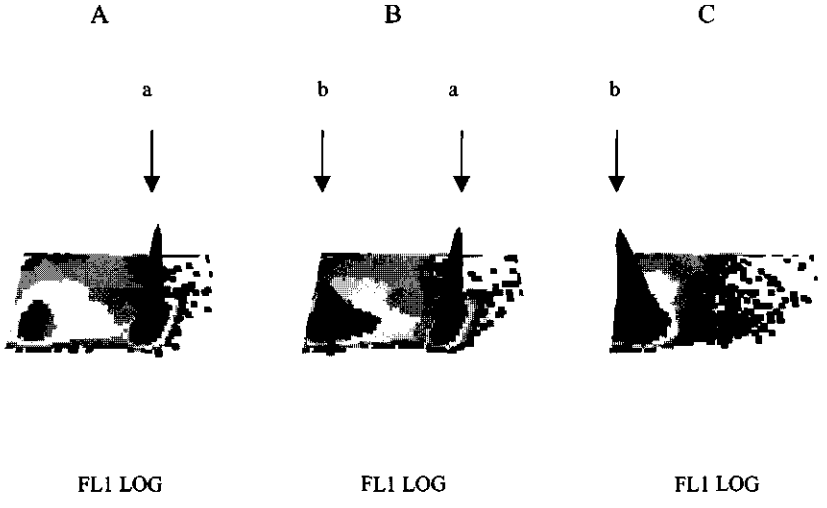

Fig. 2. Fluorescence intensity (FL1) of FITC-labeled Xcc cells in seed extracts artificially inoculated with pure suspensions of Xcc 367 (a) and / or 5053 (b). The pure suspensions were added to the cabbage seeds, extracted and incubated with the mixture of FITC-conjugated monoclonal antibodies (18G12, 2F4, and 20H6). A = 100% Xcc 367; B = 50% : 50% Xcc 367 and P5053; C= 100% P5053.

Detection of Xcc in naturally contaminated cabbage seed lots

Three naturally Xcc contaminated cabbage seed lots, with different levels of infection, were tested for the presence of Xcc cells applying the FCM technique in combination with FITC-conjugated Mabs (18G12, 2F4 and 20H6), and applying the plate count method (Table 3). FCM and the plate count method detected Xcc cells in all three samples tested. The number of Xcc cells detected by FCM and plate counts was proportional to the infection level of the seed lot (high, intermediate, and low), and it

was always higher in FCM than in plate counts. Antibody-labeled Xcc cells detected by FCM in naturally contaminated seed extract are shown in Fig. 3.

Table 3. Comparison of FCM counts and plate counts of Xcc, for similar dilutions. Three naturally contaminated cabbage seed lots, varying in contamination level, were used.

Level of Xcc in seed extracts	Seed sub-samples	Flow counts (cells ml^{-1})	Plate counts (CFU ml^{-1})
High level	1	1.0×10^7	4.8×10^3
	2	0.9×10^7	5.1×10^3
	3	1.0×10^7	7.3×10^3
average		0.9×10^7	5.7×10^3
Intermediate level	1	1.3×10^6	2.0×10^3
	2	0.5×10^6	1.1×10^3
	3	1.0×10^6	7.2×10^3
average		0.9×10^6	3.4×10^3
Low level	1	1.1×10^5	1.5×10^3
	2	2.8×10^5	0.7×10^3
	3	0.8×10^5	1.9×10^3
average		1.6×10^5	1.4×10^3

Discussion

The feasibility of applying the FCM technique in combination with Xcc-specific, FITC-conjugated Mabs (18G12, 2F4 and 20H6) for the detection and quantification of Xcc cells, especially in cabbage seed extracts, was shown in this study. The use of FCM in combination with the specific Mabs for the detection of Xcc strains performed well in pure as well as in suspensions containing both Xcc and non-pathogenic strains. These results were also confirmed by the immunofluorescence microscopy analysis. FCM appears to be a faster method compared to IF and ELISA, because the analysis of each suspension could be performed in approximately 32 minutes (incubation time and FCM analysis). FCM in combination with fluorescent monoclonal antibodies has also been shown to be a rapid and accurate method for the detection of specific Salmonella serotypes in pure suspensions. These analyses were performed within 30 minutes and the detection levels were found to be up to 10^4 cells ml^{-1} (McClelland and Pinder 1994a).

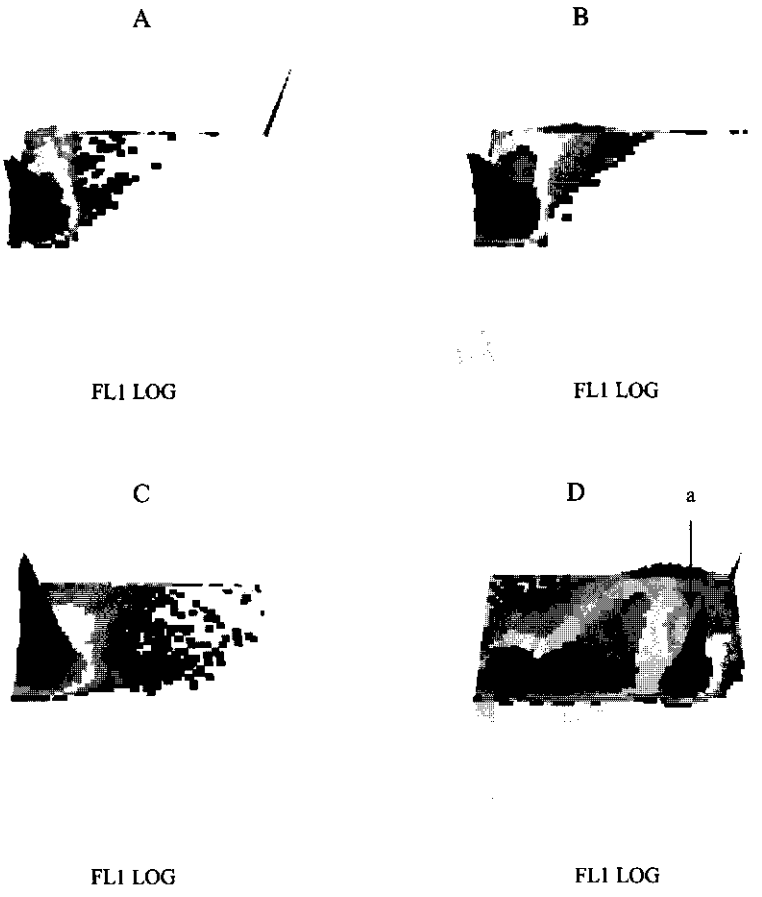

Fig. 3. Detection by FCM of Xcc cells (a) labeled with a mixture of Mabs (18G12, 2F4, and 20H6) FITC-conjugated in crude cabbage seed extract. A = Phosphate buffer (control); B = Seed extract, non-stained; C = Healthy seed extract, stained with Mabs; D = Xcc contaminated seed extract, stained with FITC-labeled Mabs. The arrow (a) indicates the population of Xcc cells.

Cabbage seed extracts contain a variety of organisms, which may interfere in the sensitivity, accuracy, and reliability of the methods that are routinely applied for the detection of Xcc. FCM could rapidly detect and quantify antibody-labeled Xcc cells and distinguish them from other microbial cells or particles present in the seed extracts, based on the high intensity of green fluorescence of the FITC-labeled cells. McClelland and Pinder (1994b) also applied FCM in combination with fluorescent antibodies to detect *Salmonella typhimurium* in eggs and milk, and concluded that this technique offers advantages of speed and sensitivity for the detection of specific pathogenic bacteria in food.

The FCM technique has been proved to perform well in several areas such as in medical, veterinary, and environmental microbiology. In the field of plant pathology, this technique is new, and may be a powerful tool when used in combination with fluorescence probes technology. In this study, FCM in combination with specific FITC-labeled monoclonal antibodies was shown to be a rapid and quantitative technique for the detection and quantification of Xcc cells in crude seed extracts. Its reliability needs to be confirmed in future research by testing more seed lots and comparing it with currently accepted routine test methods.

Acknowledgement

We thank the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES (Brasil) for provinding financial support to L. G. Chitarra during this study.

We also thank Bejo Zaden B.V., The Netherlands, for providing the Xc isolates, the seed lots, and ELISA test results.

References

Diaper, J.P., Edwards, C., Stewart-Tull, D.E.S. and Sussman, M. (1992) The detection of bacteria in aquatic environments by flow cytometry. The release of genetically modified microorganisms. In: Stewart, T.D. (ed.). *Proceedings of a symposium held under the auspices of the Federation of European Microbiological Societies.* August 29-31, 1991, Nottingham, UK. pp. 233-234.

Franken, A.A.J.M. (1992) Application of polyclonal and monoclonal antibodies for the detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds using immunofluorescence microscopy. *Netherlands Journal of Plant Pathology* **98:** 95-106.

Kusunoki, H., Kobayashi, K., Kita, T., Tajima, T., Sugii, S. and Uemura, T. (1998) Analysis of enterohemorrhagic *Escherichia coli* serotype 0157 : H7 by flow cytometry using monoclonal antibodies. *Journal of Veterinary Medical Science* **60(12):** 1315-1319.

Li, H. and Walker, D.H. (1992) Characterization of rickettsial attachment to host cells by flow cytometry. *Infection and Immunity* **60(5):** 2031-2035.

May, J.D., Branton, S.L., Pruett, S.B. and Ainsworth, A.J. (1994) Differentiation of two strains of *Mycoplasma gallisepticum* with monoclonal antibodies and flow cytometry. *Avian Disease* **38(3):** 542-547.

McClelland, R.G. and Pinder, A.C. (1994a) Detection of low levels of specific Salmonella species by fluorescent antibodies and flow cytometry. *Journal of Applied Bacteriology* **77:** 440-447.

McClelland, R.G. and Pinder, A.C. (1994b) Detection of *Salmonella typhimurium* in dairy products with flow cytometry and monoclonal antibodies. *Applied and Environmental Microbiology* **60(12):** 4255-4262.

Muirhead, K.A., Horan, P.K. and Poste, G. (1985) Flow cytometry: Present and future. *Biotechnology* **3:** 337-356.

Page, S. and Burns, R.G. (1991) Flow cytometry as a means of enumerating bacteria introduced into soil. *Soil Biol. Biochem.* **23(11):** 1025-1028.

Pinder, A.C., Purdy, P.W., Poulter, S.A.G. and Clark, D.C. (1990) Validation of flow cytometry for a rapid enumeration of bacterial concentrations in pure cultures. *Journal of Applied Bacteriology* **69:** 92-100.

Pinder, A.C. and McClelland, R.G. (1994) Rapid assay for pathogenic *Salmonella* organisms by immunofluorescence flow cytometry. *Journal of Microscopy* **176(1):** 17-22.

Porter, J., Edwards, C., Morgan, A.W. and Pickup, R.W. (1993) Rapid, automated separation of specific bacteria from lake water and sewage by flow cytometry and cell sorting. *Applied and Environmental Microbiology* **59(10):** 3327-3333.

Randhawa, P.S. and Schaad, N.W. (1984) Selective isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds. *Phytopathology* **74:** 268-272.

Schaad, N.W. (1988) Initial identification of common genera. In: Schaad, N.W. (ed.). *Laboratory guide for identification of plant pathogenic bacteria.* The American Phytopathological Society, St Paul, USA. pp. 1-15.

Schaad, N.W. (1989) Detection of *Xanthomonas campestris* pv. *campestris* in Crucifers. In: Saettler, A.W., Schaad, N.W. and Roth, D.A. (eds.). *Detection of bacteria in seed and other planting material.* The American Phytopathological Society, St. Paul, USA. pp 68-75.

Williams, P.H. (1980) Black rot: a continuing threat to world crucifers. *Plant Disease* **64:** 736-742.

Chapter 6

General discussion

Introduction

The methods applied to test plants and seeds for contamination with plant pathogenic bacteria are usually based on plating assays or on serological techniques. The conventional plate count method is used routinely and allows isolation of the target bacteria by plating plant material (Shirakawa *et al.* 1991; De la Cruz *et al.* 1992; Goszczynska and Serfontein 1998) or seed washings on selective or semi-selective media (Schaad and Donaldson 1980; Randhawa and Schaad 1994; Chang *et al.* 1990; Chang *et al.* 1991), followed by identification. This is a time-consuming process (Lange *et al.* 1993; Plihon *et al.* 1995), taking from 2 days up to one or more weeks. Serological techniques can be performed in a shorter timespan than most plating assays, however, they do not discriminate between viable and non-viable cells (except for immunofluorescence colony-staining - IFC), are time-consuming and laborious.

Flow cytometry (FCM) is a technique that has the ability to measure several parameters on thousands of individual cells within a few minutes. It also combines the advantages of microscopy and biochemical analysis in a single, highly sensitive technique for a rapid examination of numerous individual cells (Muirhead *et al.* 1985). In the field of microbiology, 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, 1994b), to distinguish between viable and non-viable bacteria (Diaper and Edwards 1994a, 1994b), to characterize bacterial DNA content (Allman *et al.* 1992; Christensen *et al.* 1993), and to characterize fungal spores (Allman 1992). Based on these successful examples, we decided to study the potential of FCM for the field of plant pathology.

This thesis describes the development and application of a combination of flow cytometry and fluorescent probes technology to detect and to assess the viability

of plant pathogenic bacteria, in particular *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) and *Xanthomonas campestris* pv. *campestris* (Xcc), in a rapid, reliable and accurate way.

Assessment of the viability of bacteria applying flow cytometry, spectrofluorometry and fluorescent probe technology

Viability is 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, and to perform several activities including DNA transcription, RNA translation, enzyme activity, reproduction and growth.

In this thesis, the viability of Cmm cells was first determined by measuring the intracellular pH (pH_{in}) as a parameter for viability (Chapter 2). This concept is based on the capability of a cell to maintain its pH gradient under conditions where the external pH is sub-optimal, i.e. pH_{in} higher than pH_{out}. For Cmm, it was shown that when the pH_{in} drops to 5.5 or below, growth is inhibited. Therefore, viable Cmm cells should maintain their pH_{in} above this pH value. *Leuconostoc mesenteroides* and *Lactobacillus plantarum* are not able to grow when their pH_{in} drops below 5.4 and 4.6, respectively (McDonald *et al.* 1990). The pH_{in} of Cmm could be determined applying the fluorescent probe 5 (and 6-)- carboxyfluorescein succinimidyl ester (cFSE). cFSE is a pH-dependent fluorescent probe which has been applied successfully in pH_{in} measurements of several Gram-positive bacteria (Breeuwer 1996). cFSE forms conjugates with aliphatic amines (proteins) in the cell and is therefore better retained within the cell than non-conjugated probes such as carboxyfluorescein (cF) and 2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) (Haughland 1996; Breeuwer *et al.* 1996). The pH_{in} of Cmm cells exposed to acid treatments (0.1, 0.2, or 0.6 mol l^{-1} of HCl for 1 hour) was determined using fluorescence spectrofluorometry, and for cells exposed to elevated temperatures (40, 45, or 50°C for 1 hour) the pH_{in} was determined using fluorescence spectrofluorometry and flow cytometry. The viability of Cmm cells was affected when cells were treated with HCl; no pH gradient could be detected ($pH_{in} = pH_{out}$) probably due to a low esterase activity in the cytoplasm, or to an increased efflux of cFSE resulting from the cell membrane damage caused by the acid treatment. Concomitantly, only a small percentage of HCl-treated cells (< 0.001%) could be recovered on plate. The spectrofluorometry

technique allows rapid measurement at the population level, however at least 10^7 viable cells ml^{-1} were needed to be able to detect a pH gradient, i.e. to get a fluorescence signal of sufficient magnitude. This indicates that the sensitivity of the technique is rather low. The observed decrease in the ability of Cmm cells to maintain a pH gradient indicated that the temperature treatments affected the viability of Cmm cells, as confirmed by the decrease in the number of CFUs observed in a plate count assay. FCM analysis could distinguish populations of heat-treated and non-treated Cmm cells based on differences in the fluorescence ratios (pH gradients) after labeling with cFSE. FCM separated Cmm cells from the background by their side and forward scatter characteristics. From the FL1/FL2 dot plots, the ratio of the green and red signals (FL1/FL2) was calculated, and based on this ratio, the intracellular pH could be calculated. The heat-treated cells had a low fluorescence ratio (no pH gradient) and could not be recovered on plates, whereas the ratio of live cells was significantly higher (pH gradient present). In practice, tomato seeds are treated with hot water (Blood 1933; Shoemaker and Echandi 1976), hydrochloric acid (*Thyr et al.* 1973; Dhanvantari 1989), or sodium hypochlorite (Shoemaker and Echandi 1976) to erradicate Cmm cells. To test the efficacy of such treatments, the conventional plate count technique is used. However, this method is time-consuming. FCM in combination with cFSE showed to be a rapid and accurate technique to test the efficacy of hot water and HCl treatments on the viability of pure cultures of Cmm. Now, the challenge is to detect Cmm cells in tomato seed extracts using the FCM technique in combination with FITC-conjugated antibodies, and consequently, to combine the detection and assessment of the viability in a single assay.

The assessment of the viability of Cmm was also evaluated applying the enzyme activity fluorescent probes Calcein acetoxy methyl ester (Calcein AM) and carboxyfluorescein diacetate (cFDA), and the nucleic acid probe propidium iodide (PI), in combination with flow cytometry (Chapter 3). The use of FCM to distinguish between viable and non-viable bacteria after labeling with cFDA, Calcein AM or PI had been reported earlier for microorganisms in food, compost extracts and seawater (Diaper and Edwards 1994a, 1994b; Magariños *et al.* 1997). In our study, heat-treated Cmm cells at 80°C for 30 minutes (non-viable) and non-treated (viable) cells were mixed in different ratios, 100/0, 80/20, 50/50, 20/80, and 0/100% respectively, to create populations varying in viability. The Cmm cells could be distinguished and

separated by FCM based on the fluorescence intensity of the cells after labeling with Calcein AM or cFDA (green fluorescence), or PI (red fluorescence). Non-treated cells showed relatively high green fluorescence levels (Calcein AM or cFDA), whereas damaged cells (heat-treated) showed high red fluorescence levels (PI). The cells were separated from the background by their side scatter characteristics. A good correlation was found between the percentage of viable Cmm cells and the FCM counts when cells were Calcein AM-stained. Surprisingly, these results differ from the results reported by Kaneshiro *et al.* (1993) and Diaper and Edwards (1994b). Their research showed the inability of Calcein AM to stain many yeast and bacterial cells, probably due to a poor accessibility of the cells for this dye. PI is not supposed to cross intact cell membranes (Alvarado-Aleman *et al.* 1996), but was in our study able to stain 18 to 56% of non-treated Cmm cells when applied as a single stain. This confirmed the idea that PI is mainly a good indicator for damaged or dead cells. The plate counts showed that the recovery of Cmm cells in the presence of Calcein AM was higher than in the presence of cFDA. This indicates that cFDA affects the viability of the cells. Also, the sorting of cells labeled with cFDA showed that only 0.6 to 5% of these cells were able to form colonies after plating, whereas 42 to 65% of sorted Calcein AM-stained cells could form colonies. As expected, PI-stained cells could not be recovered after plating.

In conclusion, the application of flow cytometry in combination with fluorescent probes was shown to be a feasible technique for assessing the viability of a pure culture of Cmm cells when cells were labeled with cFSE or Calcein AM (Chapters 2, 3).

Detection of plant pathogenic bacteria applying flow cytometry and fluorescent antibodies

FCM was evaluated for the detection of Xcc cells labeled with FITC-monoclonal antibodies in pure suspensions, in mixed suspensions with the commonly occurring saprophytic bacterium *Pseudomonas fluorescens* (Psf), in the presence of other Xanthomonads, and in crude cabbage seed extracts, artificially and naturally Xcc-contaminated. It was shown that the concentration of the FITC-labeled monoclonal antibodies (Mab) affected the sensitivity of FCM measurements. The concentration of Mab has also been reported to affect the sensitivity of serological techniques, such as

immunofluorescence microscopy (IF) (Franken 1992) and immunofluorescence colony-staining (IFC) (Van Vuurde 1997). This is based on the concept that the optimal concentration of the antibody is the one that gives the greatest discrimination between the positive cells (fluorescence staining-signal) and negative cells (non-specific binding). In this study, no cross-reactions were observed with the common saprophyte Psf, independent of the ratio Xcc/Psf tested (Chapter 4), or other Xanthomonads added to the samples (Chapter 5). FCM was able to detect and to quantify Xcc cells labeled with FITC-monoclonal antibodies in artificially and in three naturally contaminated seed lots containing different levels of Xcc infection. FCM counts were higher than plate counts due to the fact that antibodies do not discriminate between viable and non-viable cells. The detection limit for Xcc cells labeled with FITC-monoclonal antibodies using the FCM method was 10^3 CFU ml^{-1}, while the detection of *Salmonella* spp. in pure cultures, or in a complex food matrix, was shown to be reliable down to the number of 10^4 cells ml^{-1} (McClelland and Pinder 1994a, 1994b). The detection limit for plant pathogens in plant materials and environmental samples applying the conventional plating technique and the IFC method is limited to about 10^4 and 10^2 CFU ml^{-1}, respectively (Van Vuurde *et al.* 1995). However, both methods are laborious and time-consuming. In our study, FCM showed to be rapid, accurate, and is proposed to be a promising alternative method for the detection and quantification of Xcc cells in combination with Mabs. The only prerequisite for the use of antibodies is that no cross-reactions with other bacteria present in the sample occur.

Future perspectives

This thesis shows the potential of applying the FCM technique in the field of phytopathology, in particular in seed health testing. Because various economically important phytobacteria are seed-borne, seed industries have to test their seed lots for infection or contamination with bacteria. Plating assays are used routinely to detect and to assess the viability of the bacteria. Such assays take a long time and meanwhile, seed industries have to store the seeds before they can be processed and become available to the market. This results in a significant increase in costs. Therefore, a rapid, reliable and accurate method to detect and to assess the viability of plant pathogenic bacteria is highly desirable. Flow cytometry combined with

fluorescent probes technology may fulfill these requirements. The advantages and disadvantages of FCM compared to various methods are given in Table 1 for several parameters.

Table 1. Advantages and disadvantages of current methods available to detect and to assess viability of plant pathogenic bacteria.

Parameters	Plate counts	IF	ELISA	PCR	FCM
Accuracy	+	±	±	±	+
Reliability	+	±	±	+	+
Sensitivity	±	+	+	++	+
Specificity	+	±	±	-	±
Cheapness	++	+	+	±	-
Quickness	-	±	±	±	++
Labor efficiency	-	±	±	±	++
Simplicity					
Low need for technical service	++	+	+	+	±
Low complexity of sample preparation / test performance	-	±	±	±	++
Low skilled technicians	+	+	++	+	±

++ = very high (very good); + = high; ± = moderate; - = low (bad)
IF = immunofluorescence microscopy; ELISA = enzyme-linked immunosorbent assay; PCR = polymerase chain reaction; FCM = flow cytometry.
The parameters were based on:
Accuracy: precision of a test; Reliability: trustworthy; Sensitivity: the minimum amount of target detectable organisms; Specificity: degree of cross-reactivity; Cheapness: equipments and lab materials; Quickness: time required to perform the test; Labor efficiency: (++) = not much time of personal required; Simplicity: as described above.

The detection of Xcc cells in cabbage seed extract was achieved applying FCM in combination with FITC-conjugated monoclonal antibodies. However, the FCM technique should also be evaluated for the detection of FITC-labeled Xcc cells after treatment of cabbage seed lots, e.g. with hot water, HCl or chlorine, treatments applied to eradicate pathogens present. Maybe these treatments affect the surface properties of Xcc cells and consequently, may affect the interaction between antibody-cell and the FCM measurements.

FCM analysis showed that the intensity of fluorescence levels of FITC-labeled Cmm cells varied according to the amount of FITC-conjugated polyclonal antibodies that bound to the cells, making the measurement difficult to interpret. This problem is due to the variability of the morphology of Cmm cells, which vary considerably in shape and in size. However, the detection may be optimized by applying different antibodies or antibodies labeled with different fluorescent probes, or even in combination with viability staining. Since the latest generation flow cytometers are more sensitive and can measure three different fluorescence parameters, the right combination of labeled antibodies and fluorescent probes may achieve simultaneous detection and assessment of the viability of the cells in a single assay.

The assessment of the viability of Xcc cells was evaluated applying FCM in combination with the enzyme activity probes Calcein AM and cFDA, and the nucleic acid probe PI. Although these probes were already proved to perform well to assess the viability of Gram-positive bacteria, attempts to stain Xcc, a Gram-negative bacterium, were unsuccessful; probably due to the double cell membrane of Xcc being impermeable to these dyes. Nowadays, new probes have been developed to assess the viability of Gram-negative bacteria, and these should be tested for Xcc.

Unfortunately, flow cytometers are not simple instruments. As with all sophisticated measuring devices, it is important to have a basic knowledge of the underlying principles to enable a correct interpretation of the results. The quality of the sample preparation, the staining procedure, and the settings for the measurements, are as important for the precision and accuracy of the measurements as the design of the fluidic, optical, and electronic components of the instrument itself. Flow cytometers can also be equipped with flow sorting, which is used for identification and subsequent characterization of sub-populations of cells within mixed population. Flow sorting can separate positively stained cells from non-stained cells and it has the major advantage that any combination of analytical parameters can be used to set the criteria for sorting. Therefore, specialized technicians are needed for operational purposes. Nonetheless, FCM can be a feasible method for routine use to detect and to assess the viability of plant pathogenic bacteria.

References

Allman, R. (1992) Characterisation of fungal spores using flow cytometry. *Mycol. Res.* **96:** 1016-1018.

Allman, R., Hann, A.C., Manchee, R. and Lloyd, D. (1992) Characterization of bacteria by multiparameter flow cytometry. *Journal of Applied Bacteriology* **73:** 438-444.

Alvarado-Aleman, F.J., Collazo-Ponce, A. and Kumate-Rodrigues, J. (1996) Assessment of viability of yeast by flow cytometry using light green as vital dye exclusion. *Rev. Lat.-Amer. Microbiol.* **38:** 25-30.

Blood, H.L. (1933) The control of tomato bacterial canker (*Aplanobacter michiganensis* E.F.S.) by fruit pulp fermentation in the seed extraction process. *Proc. Utah Acad. Sci. Arts and Letters* **10:** 19-23.

Breeuwer, P., Drocourt, J.L., Rombouts, F.M. and Abee, T. (1996) A novel method for continuous determination of the intracellular pH in bacteria with the internally conjugated fluorescent probe 5 (and 6-)-carboxyfluorescein succinimidyl ester. *Applied and Environmental Microbiology* **62:** 178-183.

Chang, C.J, Donaldson, R., Crowley, R. and Pinnow, D. (1990) Isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds using a highly selective medium. In: Klement, Z. (ed.). *Proceedings of the 7th International Conference on Plant Pathogenic Bacteria.* Hungary. Budapest: Akadémiai Kiadó. pp. 533-539.

Chang, C.J., Donaldson, D., Crowley, M. and Pinnow, D. (1991) A new semiselective medium for the isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds. *Phytopathology* **81:** 449-453.

Christensen, H., Bakken, L.R. and Olsen, R.A. (1993) Soil bacterial DNA and biovolume profiles measured by flow-cytometry. *FEMS Microbiology Ecology* **102:** 129-140.

De la Cruz, A.R., Wiese, M.V. and Schaad, N.W. (1992) A semiselective medium for isolation of *Clavibacter michiganensis* subsp. *sepedonicus* from potato tissues. *Plant Disease* **76(2):** 830-834.

Dhanvantari, B.N. (1989) Effect of seed extraction methods and seed treatments on control of tomato bacterial canker. *Canadian Journal of Plant Pathology* **11:** 400-408.

Diaper, J.P. and Edwards, C. (1994a) Flow cytometric detection of viable bacteria in compost. *FEMS Microbiology Ecology* **14:** 213-220.

Diaper, J.P. and Edwards, C. (1994b) The use of fluorogenic esters to detect viable bacteria by flow cytometry. *Journal of Applied Bacteriology* **77:** 221-228.

Franken, A.A.J.M. (1992) Application of polyclonal and monoclonal antibodies for the detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds using immunofluorescence microscopy. *Netherlands Journal of Plant Pathology* **98:** 95-106.

Goszczynska, T. and Serfontein, J.J. (1998) Milk-Tween agar, a semiselective medium for isolation and differentiation of *Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli*. *Journal of Microbiological Methods* **32(1):** 65-72.

Haugland, R.P. (1996) Handbook of fluorescent probes and research chemicals, 6th ed. *Molecular Probes*, Inc., Eugene, Oregon, USA, 679 pp.

Kaneshiro, E.S., Wyder, M.A., Wu, Y.P. and Cushion, M.T. (1993) Reliability of calcein acetoxy methyl ester and ethidium homodimer or propidium iodide for viability assessment of microbes. *Journal of Microbiological Methods* **17:** 1-16.

Lange, H., Bavouzet, J.M., Taillandier, P. and Delorme, C. (1993) Systematic error and comparison of four methods for assessing the viability of *Saccharomyces cerevisiae* suspensions. *Biotechnology Techniques* **7:** 223-228.

Magariños, B., Romalde, J.L., Cid, A. and Toranzo, A.E. (1997) Viability of starved *Pasteurella piscicida* in seawater monitored by flow cytometry and the effect of antibiotics on its resuscitation. *Letters in Applied Microbiology* **24:** 122-126.

McDonald, L.C., Fleming, H.P., Hassan, H.M. (1990) Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. *Applied and Environmental Microbiology* **56(7):** 2120-2124.

McClelland, R.G. and Pinder, A.C. (1994a) Detection of low levels of specific Salmonella species by fluorescent antibodies and flow cytometry. *Journal of Applied Bacteriology* **77:** 440-447.

McClelland, R.G. and Pinder, A.C. (1994b) Detection of *Salmonella typhimurium* in dairy products with flow cytometry and monoclonal antibodies. *Applied and Environmental Microbiology* **60(12):** 4255-4262.

Muirhead, K.A., Horan, P.K. and Poste, G. (1985) Flow cytometry: Present and future. *Biotechnology* **3:** 337-356.

Page, S. and Burns, R.G. (1991) Flow cytometry as a means of enumerating bacteria introduced into soil. *Soil Biology and Biochemistry* **23:** 1025-1028.

Pinder, A.C., Purdy, P.W., Poulter, S.A.G. and Clark, D.C. (1990) Validation of flow cytometry for rapid enumeration of bacterial concentrations in pure cultures. *Journal of Applied Bacteriology* **69:** 92-100.

Plihon, F., Taillandier, P. and Strehaiano, P. (1995) A direct and simple method for rapid counting viable chains of *Leuconostoc* in batch cultures. *Biotechnology Techniques* **9:** 451-456.

Randhawa, P.S, and Schaad, N.W. (1984) Selective isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds. *Phytopathology* **74:** 268-272.

Schaad, N.W. and Donaldson, R.C. (1980) Comparison of two methods for detection of *Xanthomonas campestris* in crucifer seeds. *Seed Science and Technology* **8:** 383-391.

Shirakawa, T., Sasaki, T. and Ozaki, K. (1991) Ecology and control of tomato bacterial canker and detection methods of its pathogen. *Japan Agricultural Research Quarterly* **25(1):** 27-32.

Shoemaker, P.B. and Echandi, E. (1976) Seed and plant bed treatments for bacterial canker of tomato. *Plant Dis. Rep.* **60:** 163-166.

Steen, H.B., Boye, E., Skarstad, K., Bloom, B., Godal, T. and Mustafa, S. (1982) Applications of flow cytometry on bacteria: cell cycle kinetics, drug effects and quantitation of antibody binding. *Cytometry* **2:** 249-257.

Thyr, B.D., Webb, R.E., Jaworski, C.A. and Ratcliffe, T.J. (1973) Tomato bacterial canker: Control by seed treatment. *Plant Dis. Rep.* **57:** 974-977.

Van Vuurde, J.W.L., Kastelein, P. and Van der Wolf, J.M. (1995) Immunofluorescence colony-staining (IFC) as a concept for bacterial and ecological research. *EPPO Bulletin* **25:** 157-162.

Van Vuurde, J.W.L. (1997) Immunofluorescence colony-staining as a tool for sample indexing and for the determination of field thresholds for pathogenic seedborne bacteria In: Hutchins, J.D. and Reeves, J.C. (eds.). Seed *Health Testing Progress Towards the 21th Century*. Cambridge, UK. pp.165-173.

Summary

Plant pathogenic bacteria cause major economic losses in commercial crop production worldwide every year. The current methods used to detect and to assess the viability of bacterial pathogens and to test seed lots or plants for contamination are usually based on plate assays or on serological techniques. Plating methods provide information about cell viability, but are generally laborious and time-consuming. Serological techniques, such as immunofluorescence microscopy (IF) and enzyme-linked immunosorbent assay (ELISA), are much faster than most of the plating assays. However, they provide the user only with semi-quantitative information, which for various tests is not satisfactory, and they do not distinguish between viable and non-viable cells. Flow cytometry (FCM) is a rapid, reliable, and sensitive technique that has been successfully applied to detect and to assess the viability of several microorganisms in the field of veterinary science, medicine, and microbiology, and it could be worth exploring in the field of plant pathology. The research described in this thesis focused on the development of a rapid, reliable, and accurate method for the detection and assessment of viability of the seed-borne organisms *Xanthomonas campestris* pv. *campestris* (Xcc), the causal agent of black rot on cabbage, and *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), the cause of bacterial canker of tomato, by applying fluorescent probes in combination with flow cytometry or spectrofluorometry.

The viability of Cmm cells was first determined by measuring the intracellular pH (pH_{in}), as a parameter for viability, applying the fluorescent probe 5(and 6-)-carboxyfluorescein succinimidyl ester (cFSE) in combination with fluorescence spectrofluorometry or flow cytometry (Chapter 2). The growth of Cmm cells in Glucose-Nutrient-Broth medium supplemented with potassium chloride in the absence and presence of the ionophore nigericin was evaluated to determine the minimum pH_{in} value at which cells are able to grow. In the presence of nigericin (0.1 μmol^{-1}), which equilibrates the intracellular and the extracellular pH_{out} ($pH_{in} = pH_{out}$), Cmm was not able to grow at pH 5.5 and below. Therefore, viable cells should maintain their intracellular pH above this pH value. The pH_{in} of Cmm cells exposed to acid treatments, 0.1, 0.2 or 0.6 mol l^{-1} of HCl for 1 hour, was determined using

fluorescence spectrofluorometry. In HCl treated cells no pH gradient could be detected ($pH_{in} = pH_{out}$). Fluorescence microscopy revealed that these cells were poorly labeled with cFSE, either due to a low esterase activity in the cytoplasm or due to an increased efflux of cFSE resulting from the damage caused by the acid treatment. The spectrofluorometry analysis for pH_{in} measurements was not able to detect the signal of these weakly stained cells and only a small percentage of HCl treated cells (0.001%) could be recovered on plate. For cells exposed to elevated temperatures, 40, 45 or 50 °C for 1 hour, the pH_{in} was determined using cFSE in combination with flow cytometry and fluorescence spectrofluorometry. A good correlation ($r^2 \geq 0.80$) was found between the number of colony-forming units per ml (CFU ml^{-1}) determined by plate counting and the magnitude of the pH gradient (pH_{out} - pH_{in}) determined with spectrofluorometry for the heat-treated populations. However, with the spectrofluorometry technique the analysis is based on the whole cell population and the sensitivity of this technique was found to be rather low. In our experiments, cell numbers of at least 10^7 CFU ml^{-1} were needed for the analysis. Using flow cytometry, which measures fluorescence intensity of individual cells, heat-treated and non-treated Cmm cells could be distinguished based on differences in the fluorescence ratios (pH gradients) after labeling with cFSE. From the FL1/FL2 dot plots the ratio of the green and the orange signals (FL1/FL2) could be calculated (after back transformation from log to linear mode). From this ratio the intracellular pH was calculated. The heat-treated cells had a low fluorescence ratio (no pH gradient) and could not be recovered on plates, whereas the ratio of live cells was significantly higher (pH gradient present). The major advantages of flow cytometry when compared with spectrofluorometry were its sensitivity and speed, because the analysis could be performed in two hours.

In Chapter 3, the fluorescent enzyme activity probes Calcein acetoxy methyl ester (Calcein AM) and carboxyfluorescein diacetate (cFDA), and the nucleic acid probe propidium iodide (PI), were evaluated for assessing the viability of Cmm cells when applied in combination with flow cytometry. Heat-treated (80°C for 30 minutes) and viable (non-treated) Cmm cells were mixed in different ratios, 100/0, 50/50, 20/80, and 0/100% respectively, to create populations varying in viability. Non-treated and heat-treated Cmm cells labeled with Calcein AM, cFDA, PI, or combinations of Calcein AM and cFDA with PI, could be distinguished based on their

fluorescence intensity in flow cytometry analyses. Non-treated cells showed relatively high green fluorescence intensity levels, as the result of staining with Calcein AM or cFDA. Once inside the cell, Calcein AM and cFDA are cleaved (hydrolysed) by non-specific esterases to release fluorescein, a polar fluorescent compound which is retained inside the cells. Thus, the ability of the cell to accumulate fluorescein due to esterase activity is used as a parameter for viability. Damaged cells (heat-treated) showed high red fluorescence intensity levels, as the result of PI entering the cells with damaged cell membranes, intercalating into RNA and DNA. Flow cytometry allowed a rapid quantification and separation of viable Cmm cells labeled with Calcein AM or cFDA from heat-treated cells labeled with PI. The results showed a good correlation ($r^2 \geq 0.95$) between the percentage of non-treated cells present in the samples and the flow cytometry counts for Cmm cells labeled with Calcein AM or cFDA. A linear relation ($r^2 \geq 0.80$) was also found between the percentage of heat-treated cells in the samples and the flow cytometry counts for Cmm cells labeled with PI. However, when PI was applied as a single stain, it was able to stain 18 to 56% of non-treated Cmm cells. These results suggest that PI cannot be considered a good viability indicator for viable Cmm cells when applied alone. However, itt was shown to be a good indicator for dead or damaged cells. Therefore, the application of flow cytometry in combination with fluorescent probes appears to be a promising technique for assessing viability of Cmm cells in suspensions when cells are labeled with Calcein AM or the combination of Calcein AM with PI.

Flow cytometry was also evaluated for the rapid detection of Xcc cells labeled in pure suspensions and in suspensions containing mixtures of Xcc and the common saprophyte *Pseudomonas fluorescens* (Psf) with a specific FITC-labeled monoclonal antibody (Mab) (Chapter 4). The concentration of Mab affected the sensitivity of the flow cytometry measurements. This is based on the concept that the optimal concentration of Mab is the one that gives the greatest discrimination between the fluorescently stained target cells and cells stained as the result of non-specific binding. A limitation, however, is the concentration of target cells present in the samples. Xcc cells labeled with specific FITC-conjugated monoclonal antibodies could rapidly be detected at low numbers, i.e 10^3 colony-forming units per ml in pure suspensions and in suspensions containing both Xcc and saprophytic Psf cells. The detection limit for Xcc applying other serological techniques, such as

immunoflorescence microscopy (IF) and enzyme-linked immunosorbent assay (ELISA), is approximately 10^3 and 10^5 CFU ml^{-1}, respectively. A good correlation ($r^2 \geq 0.95$) was observed between the flow cytometry counts and plate counts, although flow counts were always higher than plate counts due to the fact that antibodies do not discriminate between viable and non-viable cells. The number of Psf cells, relative to the number of Xcc cells, did not interfere, neither in the flow cytometry measurements nor in plate counts. Thus, flow cytometry in combination with Xcc specific FITC-labeled monoclonal antibodies may provide a novel tool for rapid detection and quantification of this plant pathogenic bacterium.

In Chapter 5, the flow cytometry method applied to bacterial suspensions was evaluated as a tool for a rapid detection of Xcc cells, labeled with a mixture of three specific FITC-monoclonal antibodies (18G12, 2F4, and 20H6), in crude seed extracts. Flow cytometry allowed a rapid detection and quantification of Xcc cells labeled with FITC-monoclonal antibodies in both artificially and naturally Xcc-contaminated samples tested. Flow cytometry was able to detect the labeled Xcc cells in the seed extracts based on their high green fluorescence levels. No cross-reactions were observed with related Xanthomonads or other microorganisms present in artificially contaminated samples. In conclusion, the application of the flow cytometry technique in combination with specific, FITC-labeled monoclonal antibodies was shown to be a rapid and reliable alternative for the detection and quantification of Xcc cells in seed extracts.

The work described in this thesis showed that flow cytometry in combination with fluorescent probes can be a promising technique to detect and to assess viability of plant pathogenic bacteria. Nonetheless, the application of flow cytometry as a routine method to test seed lots or plants for contamination with bacteria has to be further explored, especially combining detection with viability assessment in the same assay.

Samenvatting

Wereldwijd wordt jaarlijks grote economische schade geleden in de land-en tuinbouw als gevolg van ziekten veroorzaakt door plantpathogene bacteriën. De huidige methoden om zaadmonsters of planten te testen op aanwezigheid van pathogene bacteriën zijn meestal gebaseerd op uitplaatmethoden of serologische technieken. Uitplaatmethoden geven informatie over levensvatbaarheid van cellen, maar deze zijn gewoonlijk bewerkelijk en tijdrovend. Serologische technieken, zoals immunofluorescentie microscopie (IF) of de enzyme linked immunosorbent assay (ELISA), zijn veel sneller dan de meeste uitplaatmethoden. Ze geven de gebruiker echter alleen semi-kwantitatieve informatie, wat niet altijd bevredigend is voor een aantal testen. Evenmin kan er geen onderscheid gemaakt worden tussen levensvatbare en dode cellen.

Flowcytometrie (FCM) is een snelle, betrouwbare en gevoelige methode welke met succes is gebruikt voor bepaling van aanwezigheid en levensvatbaarheid van diverse micro organismen in de veterinaire en medische wetenschap en de voedingsmicrobiologie, en zou tevens waardevol kunnen zijn in de plantenziektekunde. Het onderzoek in dit proefschrift richtte zich op de ontwikkeling van een snelle, betrouwbare en nauwkeurige methode voor detectie en bepaling van de levensvatbaarheid van de met zaad overdraagbare organismen, *Xanthomonas campestris* pv. *campestris* (Xcc), de veroorzaker van zwartnervigheid in kool, en *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), de veroorzaker van bacteriekanker in tomaat, daarbij gebruik makend van fluorescente probes in combinatie met flowcytometrie of spectrofluorometrie.

De levensvatbaarheid van Cmm cellen is in eerste instantie vastgesteld door het meten van de intracellulaire zuurgraad (pH_{in}), als parameter voor levensvatbaarheid, met behulp van het fluorescente label 5- en 6-carboxyfluoresceine succinimidyl ester (cFSE) in combinatie met spectrofluorometrie en flowcytometrie (Hoofdstuk 2). Om te bepalen bij welke minimale pH_{in} waarde Cmm cellen in staat waren te groeien werden deze gekweekt in aan-of afwezigheid van het ionofoor nigericine in glucose boullion medium met daaraan toegevoegd kaliumchloride. In aanwezigheid van nigericine ($0{,}1\mu mol^{-1}$), dat de intracellulaire en extracellulaire pH_{uit}(pH_{in}=pH_{uit}) in

evenwicht brengt, bleek Cmm volledig te worden geremd bij een intracellulaire pH van 5,5 of lager. Levensvatbare cellen dienen hun intracellulaire pH boven deze pH waarde te behouden. De pH_{in} van Cmm cellen, blootgesteld gedurende 1 uur aan 0,1, 0,2 of 0,6 mol^{-1} HCl, werd bepaald met behulp van fluorescentie spectrofluorometrie en van cellen blootgesteld aan verhoogde temperaturen (1 uur bij 40, 45 of 50 °C) met behulp van flowcytometrie en fluorescentie spectrofluorometrie. In cellen behandeld met HCL werd geen pH verschil gemeten (pH_{in}=pH_{uit}). Fluorescentie microscopie toonde aan dat deze cellen slechts geringe kleuring met cFSE gaven, enerzijds als gevolg van een lage esterase activiteit in het cytoplasma of als gevolg van een toegenomen uitlek van cFSE uit de cellen door schade ten gevolge van de zuurbehandeling. Met de spectrofluorometrie analyse voor de pH_{in} metingen was het niet mogelijk om deze zwak gekleurde cellen te meten; slechts een klein percentage met HCL behandelde cellen (0,001%) werd na uitplaten teruggevonden. De pH_{in} van cellen, blootgesteld aan verhoogde temperaturen van 40, 45 of 50 °C gedurende 1 uur, is gemeten met behulp van cFSE in combinatie met flowcytrometrie en fluorescentie spectrofluorometrie. Voor de hitte-behandelde populaties gemeten met spectrofluorometrie werd een goede correlatie ($r^2 \geq 0,80$) gevonden tussen het aantal kolonievormende eenheden per ml (KVE ml^{-1}) bepaald door uitplaten en de grootte van de pH gradient ($pH_{uit} - pH_{in}$). Bij spectrofluorometrie is de analyse gebaseerd op populaties en is derhalve de gevoeligheid van deze techniek vrij laag. Hierdoor waren in onze experimenten voor de analyses op zijn minst 10^7 KVE ml^{-1} nodig. Gebruik makend van flowcytometrie, waarmee de intensiteit van fluorescentie gemeten wordt van individuele cellen, gaf het de mogelijkheid om na labeling met cFSE hitte-behandelde van niet hitte-behandelde Cmm cellen te onderscheiden op basis van verschillen in van fluorescentieratios (pH gradienten). Aan de hand van FL1/FL2 dot plots kon de ratio van het groene en oranje signaal (FL1/FL2) worden berekend (na transformatie naar de log lineaire modus). Met behulp van deze ratio werd de intracellulaire pH berekend. De hitte-behandelde cellen hadden een lage fluorescentieratio (geen pH gradient) en konden niet worden teruggevonden na uitplaten terwijl de ratios voor levende cellen significant hoger waren (pH gradient aanwezig). De belangrijkste voordelen van flowcyrometrie in vergelijking met spectrofluorometrie bleken de gevoeligheid en snelheid, omdat de analyse in twee uur kon worden uitgevoerd.

In Hoofdstuk 3 zijn de fluorescente enzym actieve probes, calceine acetoxymethyl ester (Calceine AM), carboxyfluoresceine diacetaat (cFDA) en de nucleinezuur probe propidium jodide (PI), in combinatie toegepast om de levensvatbaarheid van Cmm cellen met behulp van flowcytometrie te evalueren. Om populaties te maken met verschillende verhoudingen levensvatbare en niet-levensvatbare cellen zijn hitte-behandelde (80 °C, 30 minuten) en onbehandelde Cmm cellen gemengd in respectievelijk de volgende verhoudingen: 100/0, 50/50, 20/80, and 0/100%. Cmm cellen gelabeld met calceine AM, cFDA, PI, of andere combinaties van calceine AM en cFDA met PI, konden onderscheiden worden op basis van fluorescentie intensiteit in flowcytometrie analyses. Onbehandelde cellen gaven een relatief hoge intensiteit van groene fluorescentie als gevolg van de kleuring met calceine AM of cFDA. Als calceine AM of cFDA eenmaal in de cel aanwezig zijn dan worden deze gesplitst (gehydrolyseerd) door niet specifieke esterases waardoor fluorescine vrijkomt dat in de cel aanwezig blijft. Het vermogen van cellen om fluorescine op te hopen als gevolg van esterase activiteit wordt daarom als een parameter voor levensvatbaarheid gebruikt. Beschadigde (hitte-behandelde) cellen toonden een hoge intensiteit van rode fluorescentie door de kleuring met PI, dat in staat is om in cellen met beschadigde celmembranen binnen te dringen, waarna binding aan DNA en RNA plaatsvindt. Flowcytometrie maakte een snelle kwantificering en scheiding mogelijk van levensvatbare Cmm cellen welke gelabeld waren met Calcein AM of cFDA en van hitte-behandelde cellen gelabeld met PI. De resultaten gaven een goede correlatie ($r^2 \geq 0,95$) tussen het percentage niet-behandelde cellen aanwezig in de monsters en de flowcytometrie tellingen voor Cmm cellen gelabeld met Calcein AM of cFDA. Een lineare relatie ($r^2 \geq 0,80$) werd ook gevonden tussen het percentage hitte-behandelde cellen in de monsters en in de FCM tellingen van Cmm cellen gelabeld met PI. Echter, indien PI als een enkelvoudige kleuring werd toegepast, dan werden nog 18-56 procent van de levensvatbare niet-behandelde Cmm gekleurd. Deze resultaten suggeren dat PI geen goede indicator is voor levensvatbaarheid wanneer het als enkelvoudige kleuring wordt toegepast. Aangetoond is dat PI wel een goede indicator is voor dode of beschadigde cellen. De toepassing van flowcytometrie in combinatie met fluorescerende labels lijkt daarom een veelbelovende techniek voor de bepaling van de levensvatbaarheid van Cmm

cellen in suspensies na labeling met calceine AM of de combinatie van calceine AM met PI.

Flowcytometrie is ook getoetst voor de snelle detectie van Xcc cellen welke waren gelabeld met een FITC-geconjugeerd monoclonaal antilichaam (Mab) in suspensies van reincultures en in suspensies die mengsels bevatten van Xcc en de algemeen voorkomende saprofiet *Pseudomonas fluorescens* (Psf) (Hoofdstuk 4). De concentratie van Mab had invloed op de gevoeligheid van de metingen in de flowcytometer. De optimale concentratie van Mab is de concentratie die het grootste onderscheid geeft tussen de gekleurde fluorescente doelcellen en cellen gekleurd als gevolg van niet specifieke binding. Een beperking is echter wel de concentratie van doelcellen die aanwezig zijn in een monster. Xcc cellen gelabeld met specifieke FITC-geconjugeerde monoclonale antilichamen kunnen snel worden gedetecteerd bij lage aantallen, d.w.z. 10^3 kolonievormende eenheden per ml in reincultures en in suspensies die zowel Xcc als Psf cellen bevatten. De detectielimiet voor Xcc bij toepassing van serologische technieken zoals immunofluorescentie microscopie (IF) of de enzyme-linked immunosorbent assay (ELISA), is respectievelijk 10^3 en 10^5 KVE ml^{-1}. Er werd een goede correlatie ($r^2 \geq 0.95$) gevonden tussen flowcytometrie tellingen en het aantal gevonden kolonies na uitplaten hoewel de flowcytometrie resultaten altijd hoger uitvielen dan de tellingen na uitplaten. Dit lijkt het gevolg te zijn van het feit dat de antilichamen geen onderscheid maken tussen levensvatbare en niet-levensvatbare cellen. Het aantal Psf cellen ten opzichte van het aantal Xcc cellen had geen invloed op de flowcytometrie bepalingen en de tellingen na uitplaten. Flowcytometrie heeft derhalve in combinatie met Xcc-specifiek gelabelde FITC-gelabelde (monoclonale) antilichamen potentie als methode voor snelle detectie en kwantificering van deze plantpathogene bacterie.

In Hoofdstuk 5, werd de flowcytometrie methode geëvalueerd als methode voor detectie van Xcc cellen in ruwe zaadextracten, dit na labeling met een mengsel van drie specifieke FITC-geconjugeerde monoclonale antilichamen (18G12, 2F4 en 20H6). Flowcytometrie maakte een snelle detectie en kwantificering van Xcc cellen mogelijk in monsters welke natuurlijk of kunstmatig besmet waren met Xcc. Detectie van Xcc cellen in de zaadextracten was gebaseerd op hun sterke groene fluorescentie, die duidelijk te onderscheiden was van de achtergrond. Er werden geen kruisracties met verwante Xanthomonaden of andere micro-organismen waargenomen in

kunstmatig besmette monsters. Hieruit kan worden geconcludeerd dat de toepassing van de flowcytometrie in combinatie met specifiek FITC-gelabelde monoclonale antilichamen een snel en betrouwbaar alternatief kan zijn voor de detectie en kwantificering van Xcc cellen in zaadextracten.

Algemeen kan worden geconcludeerd dat flowcytometrie in combinatie met fluorescente probes een veelbelovende techniek is om plantpathogene bacteriën aan te tonen en hun levensvatbaarheid vast te stellen. De toepasbaarheid van flowcytometrie als routine methode voor het testen van zaadmonsters of planten zal verder moeten worden onderzocht, met name de combinatie van detectie en bepaling van levensvatbaarheid in dezelfde test.

Curriculum vitae

Luiz Gonzaga Chitarra was born on December 13, 1960 in Lavras, State of Minas Gerais (MG), Brazil. In 1978 he received his certificate for the secondary education level in Volta Redonda, State of Rio de Janeiro (RJ). Between August 1979 and December 1980, he studied Mechanic Engineering at the "Pontifícia Universidade Católica de Petrópolis" (PUC) in Petrópolis (RJ). In January 1981, he moved to Tucson, State of Arizona (AZ), United States of America, to continue his studies on Mechanic Engineering. In August 1982, he changed his major to Agronomy, and on May 13 1984, he received his Bachelor Degree in Science of Agriculture. He returned to Brazil and for the following 9 years he worked for private companies, such as "Duratex S.A" in Campinas, State of São Paulo (SP), and for "Bioplanta Technologia de Plantas S.A", in Paulínia (SP).

In 1993 he decided to continue his studies and joined the Department of Phytopathology of the Federal University of Lavras (UFLA), in Lavras (MG), under supervision of Prof. Dr. José da Cruz Machado. He was awarded with a master's degree in 1996.

In 1996, he was granted with a 4 year-fellowship by the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES - Brasil), and in December 1996 he was accepted as a Ph.D student by the Department of Food Technology and Nutritional Sciences of the Wageningen University (WU), in Wageningen, The Netherlands. The work leading to his Ph.D thesis was performed at both the Wageningen University and Plant Research International.

List of Publications

Chitarra, L.G., Machado, J.C., Vieira, M.G.G.C., Silva, C.M. (1997) Desempenho de sementes de algodoeiro (*Gossypium hirsutum* L.) em função do tempo de duração do deslintamento com ácido sulfúrico. *Revista Ciência e Agrotecnologia* **21(4):** 425-435.

Chitarra, L.G. Chitarra, A.B., Machado, J.C., Menezes, J.B. (1998) Teores de açúcares neutros, ácidos urônicos e condutividade elétrica do exsudato de sementes de algodoeiro (*Gossypium hirsutum* L.), em função do tempo de duração do deslintamento químico. *Brazilian Archives of Biology and Technology* **41(1):** 129-143.

Chitarra, L.G., Breeuwer, P., Van den Bulk, R.W. and Abee, T. (2000) Rapid fluorescence assessment of intracellular pH as a viability indicator of *Clavibacter michiganensis* subsp. *michiganensis*. *Journal of Applied Microbiology* **88:** 809-816.

Chitarra, L.G., Machado, J.C., Chitarra, G.S., Vieira, M.G.G.C. Efeito do deslintamento químico de sementes de algodoeiro (*Gossypium hirsutum* L.) sobre o nível de ocorrência de *Colletotrichum gossypii* e desenvolvimento do fungo em exsudato de sementes deslintadas. Submitted.

Chitarra, L.G., Breeuwer, P., Abee, T. and Van den Bulk, R.W. The use of fluorescent probes to assess viability of the plant pathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* by flow cytometry. Submitted.

Chitarra, L.G. and Van den Bulk, R.W. New and current techniques to detect and to assess the viability of plant pathogenic bacteria. Submitted.

Chitarra, L.G., Langerak, C.J., Bergervoet, J.H.W. and Van den Bulk. Detection of *Xanthomonas campestris* pv. *campestris* in seed extracts of *Brassica* sp. applying fluorescent antibodies and flow cytometry. Submitted.

Chitarra, L.G., Bergervoet, J.H.M., Abee, T. and Van den Bulk. Detection of the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* applying fluorescent antibodies and flow cytometry. Submitted.