Immunofluorescence microscopy and dilution-plating for the detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds - methods to determine seed health and seed infection

Promotor: Dr. Ir. J. Dekker
emeritus hoogleraar in de Fytopathologie

A.A.J.M. Franken

Immunofluorescence microscopy and dilution-plating for the detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds - methods to determine seed health and seed infection

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op dinsdag 13 oktober 1992
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

ISN: 551522

Contents

NN08201, 1544

Stellingen

1. De conclusie van Schaad (1983) dat immunofluorescentie microscopie (IF) niet gebruikt dient te worden voor zaaizaad certificering, doet geen recht aan het feit dat IF bij uitstek geschikt is om de gezondheid van zaadpartijen te voorspellen.

   Schaad, N.W., 1983. Correlation of laboratory assays for seedborne bacteria with disease development. Seed Science and Technology 11: 877-883.

   Van Vuurde, J.W.L., Franken, A.A.J.M., Birnbaum, Y. & Jochems, G., 1991. Characteristics of immunofluorescence microscopy and of dilution-plating to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed lots and for risk assessment of field incidence of halo blight. Netherlands Journal of Plant Pathology 97: 233-244.

   Dit proefschrift.

2. Het feit dat het succes van uitplaatmethoden voor Xanthomonas campestris pv. campestris afhankelijk is van de onderzochte zaadpartij, het uitplaatmedium en de gebruikte extractiemethode, wordt waarschijnlijk veroorzaakt door de aanwezigheid van antibiotische stoffen in en op het zaad.

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

   Dit proefschrift.

3. Het maken van een kunstmatig onderscheid tussen isolaten van Xanthomonas campestris pv. campestris en Xanthomonas campestris pv. amoraciae, beiden pathogenen van kruisbloemigen, kan er toe leiden dat zaadpartijen ten onrechte worden aangemerkt als zijnde "vrij van zwartnervigheid".

4. Het gebruik van een zogenaamde 'dot-blot immunoassay' voor het identificeren van verdachte kolonies m.b.v. monoklonale antistoffen, is t.o.v. andere serologische technieken zoals IF aantrekkelijk vanwege de snelheid en de, soms, verminderde kans op 'vals-negatieve' reacties.

   Dit proefschrift

5. Gezien het feit dat de immunofluorescentie microscopie (IF) een officiële methode is van de AOAC ("Association of Official Analytical Chemists") voor de detectie van Salmonellae in voedselprodukten, is het - gezien de mogelijkheden tot automatisering - opmerkelijk dat deze techniek tot op heden slechts op beperkte schaal voor dit doel gebruikt wordt.

   Thomason, B.M., 1981. Current Status of Immunofluorescent Methodology for Salmonellae. Journal of Food Protection 44: 381-384.

6. Bij het toetsen van zaadpartijen op aanwezigheid van ziekteverwekkers wordt de laboratorium methode, wat betreft het aantal te toetsen zaden en submonsters, onvoldoende afgestemd op de geaccepteerde besmettingsniveaus in diverse landen en de gevoeligheid van de techniek.

Shu-Geng, Campbell, R.N., Carter, M., Hills, F.J., 1983. Quality-Control Programs for Seedborne Pathogens. Plant Disease 67: 236-242.

7. Bij het keuren van plantemateriaal op virusbesmetting m.b.v. ELISA ('enzyme-linked immunosorbent assay'), dient de extinctiewaarde die gebruikt wordt om een produkt af te keuren niet alleen afhankelijk gesteld te worden van de spreiding in extinctiewaarden van het gezond materiaal, maar ook van de resultaten behaald met andere, gevoeligere technieken zoals ISEM ('immunosorbent electron microscopy').

Sutula, C.L., Gillet, J.M., Morrissey, S.M., Ramsdell, D.C., 1986. Interpreting ELISA data and establishing the positive-negative threshold. Plant Disease 70: 722-726.

8. Aan instellingen aangesloten bij ISTA (International Seed testing Association) zouden kwaliteitseisen gesteld dienen te worden, die in overeenstemming zijn met GLP ("Good-Laboratory Practice") standaarden; dit zal het succes en het nut van internationaal vergelijkend onderzoek vergroten.

9. Reorganisaties, uitgevoerd onder het mom van 'verhoging van de doelmatigheid' onder verantwoordelijkheid van het Ministerie van Landbouw, Natuurbeheer en Visserij, dienen achteraf getoetst te worden op het bereiken van deze doelmatigheid; dat is pas échte doelmatigheid.

10. Bezuinigingen die momenteel opgelegd worden aan het veredelings- en reproduktieonderzoek stroken niet met de behoefte van de maatschappij om binnen afzienbare tijd het gebruik van bestrijdingsmiddelen terug te brengen.

11. De niet-opwindende verslaglegging van wielerwedstrijden door Nederlandse televisiecommentatoren leidt er toe dat men zich opwindt, zelfs tijdens niet-opwindende wedstrijden.

12. Vincent van Gogh's krankzinnigheid in 1890 hangt mogelijk samen met het feit dat hij niet in 'zijn' Zundert kon zijn.

A.A.J.M. Franken

Stellingen behorende bij het proefschrift: "Immunofluorescence microscopy and dilution-plating for the detection of Xanthomonas campestris pv. campestris in crucifer seeds - methods to determine seed health and seed infection".

Wageningen, 13 oktober 1992

Abbreviations used in this thesis

BSCAA: basal starch cycloheximide agar with nitrofurantoin and vancomycin and methyl green; a semi-selective medium for *X. c.* pv. *campestris*

CS20ABN: medium with starch, bacitracin, neomycin and cycloheximide; a semi-selective medium for *X. c.* pv. *campestris*

DBI: dot-blot immunoassay

EIA: enzyme immunoassay

ELISA : enzyme-linked immunosorbent assay

EPS : extracellular polysaccharide

FS medium : medium with starch, trimethoprim, cephalexin, cycloheximide, methyl green (and gentamicin); a semi-selective medium for *X. c.* pv. *campestris*

IB : immunoblotting

IF : immunofluorescence microscopy

LPS : lipopolysaccharide

MCA: monoclonal antibody

NSCA : Nutrient starch cycloheximide agar; a plating medium for *X. c.* pv. *campestris*

NSCAA: NSCA with nitrofurantoin and vancomycin; a semi-selective medium for *X. c.* pv. *campestris*

ODD : Ouchterlony double diffusion

PBS: phosphate-buffered saline

PCA: polyclonal antiserum/antibodies

RFLP : restriction fragment length polymorphism

SDS-PAGE : sodium-dodecyl-sulphate polyacrylamide gel electrophoresis

SM-medium : starch-methionine agar, with methyl violet 2B, methyl green, trace element solution, triphenyltetrazolium chloride and cycloheximide; a semi-selective medium for

X. c. pv. *campestris*

SMA-medium: SM-agar with cephalexin and nitrofurantoin; a semi-selective medium for *X. c.* pv. *campestris*

SX- medium: medium with starch, methyl violet 2B, methyl green and cycloheximide; a semi-selective medium for *X. c.* pv. *campestris*

YDC-medium : yeast-dextrose-carbonate medium

Chapter 1. Introduction

Introduction

Black rot, caused by the bacterium *Xanthomonas campestris* pv. *campestris* (*X. c.* pv. *campestris*), is one of the most threatening diseases of crucifers. The bacterium attacks all cultivated *Brassica* spp., radishes and numerous weeds (Williams, 1980). The black rot disease was first recorded in 1891 and the pathogen was described in 1895 (Pammel, 1895). In the Netherlands, the appearance of the disease was first reported by Van Hall (1900). The black rot disease is now widely distributed in parts of Africa, Asia, Australasia and Oceania, Europe, North America, Central America, West Indies and South America (Hayward and Waterston, 1965). Harding et al. (1904) were the first to report that the bacterium was seed-borne.

The bacterium may be spread to adjacent plants by windblown rain and mechanical means, and to a limited extent by insects (Shelton and Hunter, 1985; Williams, 1980). In the field, plants are usually infected through the hydathodes (Cook et al., 1952a). Potential sources of inoculum are cruciferous weeds (Kuan et al., 1986), soil-borne residues of cruciferous plants (Schultz and Gabrielson, 1986), but probably most importantly infected seeds.

Seed infection may take place through systemic infection of the mother plant. However, severely infected seed plants may be killed without flowering, and therefore usually they are of no importance in seed infection. If the disease appears just before seed maturation the plant will be important for seed infection. The organism advances from the xylem of the vascular bundles in the seed pod walls into that of the funiculus and occasionally that of the seed coat. In most cases infection is confined to the funiculus which commonly dries down and adheres to the seed. In some instances seeds may be surface-contaminated during the threshing process; organisms are then released from infected pods and aborted seeds (Cook et al., 1952b). Control measures are the application of crop rotation varying from 1 to 5 years and the use of proper cultural practices (Williams, 1980). However, the use of resistant cultivars and healthy seed are most important. The availability of good resistance genes is limited. There is still a need to find new sources of resistance, because

the use of the few resistance genes available has undoubtedly narrowed the genetic base of the hybrid crucifers (Williams, 1980). Moreover, there is a need for resistance at the seedling stage besides the mature plant resistance which is already present in some cultivars (Hunter et al., 1987). Coyne and Schuster (1983) argued that emphasis should only be placed on selection of plants with no or reduced seed-transmission of bacteria, since seed is often the source of inoculum for the emerging crop.

The use of 'healthy' seeds is another very important way to control black rot and to ensure seed quality. Healthy seeds may be obtained by application of seed treatments. However, as well as possibly not being fully effective in eliminating the pathogen from the seed, these treatments may seriously affect the germination and cause phytotoxicity (Humaydan et al., 1980). After the seed has been treated, a check on the efficacy of the treatment and the germination of the seed lot is always necessary.

To determine whether the seed lots are 'healthy' or only infected at an acceptable low level, seed lots have to be indexed for the presence of *X. c.* pv. *campestris*. Therefore, reliable, specific and sensitive methods to detect the pathogen are necessary. For *X. c.* pv. *campestris* several detection methods have been published. Some methods are based on indexing of seedlings for appearance of specific symptoms after germination (Srinivasan et al., 1973). In other methods seeds are plated on media containing starch. When, after incubation, seeds are surrounded by starch-hydrolyzing colonies they should be recorded as positive after proper confirmation of the identity of suspected colonies (Schaad and Kendrick, 1975). More recently assays have been developed based on the plating of (dilutions of) seed washings upon semi-selective media containing starch (Chang et al., 1991). In these plating assays suspected colonies should also be confirmed by other tests. Besides plating assays, a sensitive serological test such as immunofluorescence microscopy (IF) can also be used for direct detection of *X. c.* pv. *campestris* in seed washings (Schaad and Donaldson, 1980). Schaad and Donaldson (1980) obtained good results with IF and the correlation with plating results was high. However, in more recent publications the correlation between results obtained by IF and plating assays was found to be poor, i.e. with IF more seed lots were usually found positive than with dilution-plating (Schaad, 1982, 1983). In these publications the cell

counts in IF were not given, although they are important. By relating cell counts in IF to the number of positive isolations of *X. c.* pv. *campestris* in plating assays, the poor correlation between IF and plating assays could be explained and ways to improve the correlation between IF and dilution-plating could be found. So far, IF studies have only been carried out with polyclonal antibodies. No investigations have been carried out on the use of monoclonal antibodies in IF for *X. c.* pv. *campestris*. Their use may give information on the significance of cross-reactions in IF as compared to quality-tested polyclonal antibodies. Moreover, with monoclonal antibodies IF tests could become more standardized and reliable because of the constant quality (homogeneity) of these antibodies.

The aim of the research described in this thesis was to analyse important characteristics of IF and dilution-plating assays, and to improve their use for the identification and detection of *X. c.* pv. *campestris* from crucifer seeds. The literature on detection and identification of *X. c.* pv. *campestris* is reviewed in chapter 2.

Several seed washing procedures and plating media are known for the plating assays. The question whether seed lots will be found positive or negative often depends on the seed lot tested, medium and extraction method used (ISTA, 1988). To find out whether these interactions could be explained by a lack of standardization, several steps of the plating assays were evaluated using accurate prescriptions of the assay. The results of this evaluation are given in chapter 3.

Specific antibodies are needed for application of serological tests to detect and identify *X. c.* pv. *campestris*. A prerequisite for the use of antibodies is that they preferably do not cross-react with other bacteria present on the seed. Therefore, antibodies should be tested for specificity prior to use. Chapter 4 discusses the production of monoclonal antibodies and the reaction of polyclonal and monoclonal antibodies with strains of *X. c.* pv. *campestris*, other pathovars of *Xanthomonas campestris* and non-related pathogens and saprophytes.

To find out whether the antibodies give different reactions when applied to seed washings as compared to testing pure cultures, several seed lots were tested with monoclonal and polyclonal antibodies in IF. The results are described in chapter 5.

To study the correlation between dilution-plating and IF assays a large set of seed lots needs to be tested by both methods. In this way information on the sensitivity as well as the advantages and disadvantages of both tests will become clear. The results of such a study are described in chapter 6.

Chapter 7 reviews important characteristics of serological techniques such as sensitivity and specificity and deals with the problems occurring when serological techniques are used for detecting seed-borne bacteria in combination with dilution-plating. Possible solutions to the problems mentioned are also given.

In chapter 8 the overall results reported in this thesis are discussed in view of recent developments. General conclusions on the detection of *X. c.* pv. *campestris* with plating assays and IF are given.

References

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.

Cook, A.A., Walker, J.C. and Larson, R.H., 1952a. Studies on the disease cycle of black rot of crucifers. Phytopathology 42: 162-167.

Cook, A.A., Larson, R.H. and Walker, J.C., 1952b. Relation of the black rot pathogen to cabbage seed. Phytopathology 42: 316-320.

Coyne, D.P. and Schuster, M.L., 1983. Genetics of and breeding for resistance to bacterial pathogens in vegetable crops. HortScience 18: 30-36.

Harding, H.A., Stewart, M.C. and Prucha, M.J., 1904. Vitality of the cabbage black rot germ on cabbage seed. New York (Geneva) Agriculture Experiment Station Bulletin 251: 177-194.

Hayward, A.C. and Waterston, J.M., 1965. C.M.I. Description of pathogenic fungi and bacteria no. 47. *Xanthomonas campestris*.

Humaydan, H.S., Harman, G.E., Nedrow, B.L. and DiNito, L.V., 1980. Eradication of *Xanthomonas campestris*, the causal agent of black rot, from *Brassica* seeds with

antibiotics and sodium hypochlorite. Phytopathology 70: 127-131.

Hunter, J.E., Dickson, M.H. and Ludwig, J.W., 1987. Source of resistance to black rot expressed in young seedlings and adult plants. Plant Disease 71: 263-266.

ISTA, 1988. Report from the 19th International Seminar on Seed Pathology, 1987, Wageningen, the Netherlands, pp. 13-14. International Seed Testing Association, Zürich, Switzerland.

Kuan, T-L, Minsavage, G.V. and Schaad, N.W., 1986. Aerial dispersal of *Xanthomonas campestris* pv. *campestris* from naturally infected *Brassica campestris*. Plant Disease 70: 409-413.

Pammel, L.H., 1895. Bacteriosus of rutabaga (*Bacillus campestris* n. sp.). Iowa Agriculture Experiment Station Bulletin 27.

Schaad, N.W., 1982. Detection of seedborne bacterial plant pathogens. Plant Disease 66: 885-890.

Schaad, N.W., 1983. Correlation of laboratory assays for seedborne bacteria with disease development. Seed Science and Technology 11: 877-883.

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

Schaad, N.W. and Kendrick, R., 1975. A qualitative method for detecting *Xanthomonas campestris* in crucifer seed. Phytopathology 65: 1034-1036.

Schultz, T. and Gabrielson, R.L., 1986. *Xanthomonas campestris* pv. *campestris* in Western Washington crucifer seed fields: occurrence and survival. Phytopathology 76: 1306-1309.

Shelton, A.M. and Hunter, J.E., 1985. Evaluation of the potential of the flea beetle *Phyllotreta cruciferae* to transmit *Xanthomonas campestris* pv. *campestris*, causal agent of black rot of crucifers. Canadian Journal of Plant Pathology 7: 308-310.

Srinivasan, M.C., Neergaard, P. and Mathur, S.B., 1973. A technique for detection of *Xanthomonas campestris* in routine seed health testing of crucifers. Seed Science and Technology 1: 853-859.

Van Hall, C.J.J., 1900. Twee Bacteriënziekten. Tijdschrift over Plantenziekten 6:

169-177.

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

Chapter 2. A review of the literature

A review of the literature

Many measures for the control of black rot disease are based on its seed-borne nature. Smith (1920) advised the use of seed derived from healthy plants, and this advise has been taken by many research workers.

Different seed treatments have been considered by research workers for obtaining healthy seeds. Table 1 shows different kinds of seed treatments which have been reported for control of *X. c.* pv. *campestris*. Although, depending on the *Brassica* spp., cultivar, seed lot and formulation or type of application, seed treatments may be effective for eradication of the pathogen from crucifer seeds, they may have serious drawbacks. Phytotoxicity may arise and seed lots may also be affected in either their ability to germinate or their germination rate. It is therefore advisable to test the sensitivity of seed lots for specific seed treatments before large scale treatment (e.g. Harman et al., 1987). However, care should be taken since adverse effects of seed treatments may only become evident a long time after the treatment (Schultz et al., 1986).

Routine treatment of seed lots without prior knowledge of the health status of the seed lot (infected or not infected) is highly inefficient. Moreover, some seed treatments such as those using antibiotics are not always suitable because of the risk of resistance against these antibiotics. Certain chemicals are also unsuitable as they are detrimental to the environment.

Possible deleterious effects of seed treatments on seed quality makes it advisable to test the need for such a treatment by initially indexing seed lots for *X. c.* pv. *campestris*. Treatment should be avoided unless absolutely necessary, in which case its effect should be evaluated. There are few alternatives to the traditional hot water treatment. Other non-chemical seed treatments apart from aerated steam treatments are not yet known for *X. c.* pv. *campestris* in crucifer seeds. Some of these, e.g. radiation of seeds (Hankin and Sands, 1977), may be worth investigating.

However, even when seed lots do not have to be treated or resistant cultivars are used, detection methods are needed to determine the health status of the seed.

Table 1. A summary of seed treatments reported for *Xanthomonas campestris* pv. *campestris*.

Treatment	Special remarks	References
Physical methods		
Hot water treatment	Reduction of germination and seed vigour may occur, treatment often fails to eradicate *X. c.* pv. *campestris*	Clayton (1924), Walker (1952), Humaydan et al. (1980), Schaad et al. (1980), Sharma (1981), Lin (1981), Shekhawat et al. (1982), Schultz et al. (1986), Aveling and Robbertse (1990)
Aerated steam	Advantages over hot water treatments are that it avoids damage to the seeds	Navaratnam et al. (1980)
Chemical methods		
Acidified cupric acetate	*X. c.* pv. *campestris* was successfully eradicated from crucifer seeds. Reduction of germination was dependent on plant cultivar, therefore treatment was not advised for routine use.	Schaad et al. (1980), Lin (1981)
Acidified zinc sulfate	This treatment was reported to be as good or better than acidified cupric acetate, calcium hypochlorite or hot water treatments	Huang and Lee (1988)
Antibiotics chloramphenicol	Treatment successfully controlled the pathogen without reducing germination or causing phytotoxicity	Aveling and Robbertse (1990)

(continued on next page)

Table 1 (continued).

kasugamycin	No complete disinfection of seeds, phytotoxicity problems may be found	Aveling and Robbertse (1990)
streptomycin	No complete disinfection of seeds, phytotoxicity may be found and germination may be reduced	Klisiewicz and Pound (1961), Humaydan et al. (1980), Aveling and Robbertse (1990)
tetracyclines	No complete disinfection of seeds, phytotoxicity may be found, germination may be reduced	Klisiewicz and Pound (1961), Humaydan et al. (1980), Aveling and Robbertse (1990)
vancomycin	No complete disinfection of seeds, phytotoxicity less than for tetracyclines or streptomycin, or absent	Humaydan et al. (1980), Aveling and Robbertse (1990)
combinations of antibiotics: streptomycin + tetracyclines (including chlor- and oxy-tetracyclines)	Phytotoxicity may become evident in seedlings. Generally good control of black rot.	Klisiewicz and Pound (1961), Sharma (1981), Shah et al. (1985), Kishun (1984)
Mercuric chloride	Unsafe for general use (generally not allowed for use), not effective for control of black rot	Walker and Tisdale (1920), Clayton (1924, 1925), Klisiewicz and Pound (1961)
Calcium hypochlorite (slurry treatment)[1]	No immediate reduction of germination. However, reduction of germination was noted in some seed lots six months after treatment	Schultz et al. (1986)

(continued on next page)

Table 1 (continued).

Nyolate (a formulation with sodium chlorite and lactic acid)	Phytotoxicity depended on formulation and crop tested, seeds of broccoli and cauliflower were easily damaged by Nyolate treatments	Harman et al. (1987)
Other combinations		
Hot water treatment followed by streptomycin/(chlor)tetracycline treatment	Seeds were sown in 2% formalin treated soil and good control was observed	Bhardwaj et al. (1987)
Streptomycin/(chlor)tetracycline treatment followed by sodium hypochlorite treatment	Phytotoxicity problems may be overcome, although under some field conditions a phytotoxic response was still noted, germination may also be affected	Humaydan et al. (1980), Schultz et al. (1986), Harman et al. (1987)
Streptomycin treatment followed by sodium hypochlorite treatment	No reduction in rutabaga stands or phytotoxicity in laboratory or field test noted	McKeen (1981)
Streptomycin + chlortetracycline + captan	No adverse effects on germination of seeds reported	Shekhawat et al. (1982)

[1]All other treatments are generally applied through soaking seeds in the chemical.

Although it is not possible to determine if a seed lot is free from infected or infested seeds, it is possible to certify that a seed lot contains less than a specified level of infection (Schaad, 1982a). To determine this 'specified level of infection' reliable and sensitive detection methods are needed. Estimating disease on the basis of inspection in the field or even under climate-conditioned glasshouses may give misleading results since the systemic nature of seedborne *X. c.* pv. *campestris* plus cool temperatures in the spring often delay symptoms, making detection virtually impossible (Williams, 1980; Schaad, 1982a). Optimum growth conditions for the host are not optimal for development of black rot symptoms. Symptoms masked by holding plants at 15-20 °C may quickly become obvious when the temperature rises to 25-30 °C. Since temperatures are generally low during the vegetative growth stage of crucifer seed plants diseased plants are not always easily detected (Schaad, 1982a). Therefore detection methods are often based on laboratory tests performed under optimum conditions for disease and/or pathogen development. Several methods are described for detecting *X. c.* pv. *campestris* in crucifer seeds, viz. seedling-, plating-, serological assays, and combinations of these assays.

Seedling assays

The earlier methods described for detecting *X. c.* pv. *campestris* in seed lots were the so-called seedlings assays. Shackleton (1962) described a method, in which seedlings were germinated in boxes. At 22 °C seedlings infected with *X. c.* pv. *campestris* showed a progressive collapse, blackening and dying within 8-18 days of sowing.

Srinivasan et al. (1973) also developed a seedling assay for detection of *X. c.* pv. *campestris*. Seeds were treated with an antifungal antibiotic and planted on water agar. Within 8-18 days of sowing, infected seedlings showed symptoms typical of the black rot pathogen as mentioned above. *X. c.* pv. *campestris* was isolated from diseased tissues and pathogenicity proved. Bucha (1977) reported that *Bacillus* spp., *Erwinia carotovora* and *E. herbicola*, and *Pseudomonas* spp. could disturb the test reported by Srinivasan et al. (1973) and cause similar symptoms to *X. c.* pv. *campestris*. Moreover, the method developed by Srinivasan et al. cannot identify *X. c.* pv. *campestris* in aborted seeds, since

germination is required.
Since seedling assays are usually less suited for screening large numbers of seeds and seed lots, they have generally been replaced by other assays, such as plating assays.

Plating assays

More recent methods are based on direct plating of seeds or plating of liquid, derived from seed washings (soaking or shaking of seeds in a liquid solution) on media. The plating methods are summarized in table 2, as well as the media mentioned in the references cited. Schaad and White (1974) reported the development of the SX (starch-*Xanthomonas*) medium suited for selective isolation of *X. c.* pv. *campestris* from soil. This medium, primarily based on the utilization of starch by *X. c.* pv. *campestris* in a medium with cycloheximide, was later also used for isolation of *X. c.* pv. *campestris* from seed (Schaad and Kendrick, 1975). SX agar is generally not advised for plating of seed washings (Schaad, 1989), due to its low recovery of *X. c.* pv. *campestris*. Chun and Alvarez (1983) developed a starch-methionine (SM) medium which was developed for isolation of *X. c.* pv. *campestris* from infected cabbage leaves and plant debris. The medium was based on the SX medium and the minimal nutritional requirements for the genus *Xanthomonas*. The SM medium was later used for isolation of *X. c.* pv. *campestris* from seed washings (Tze-Chung and Hsu, 1987a, 1987b). Tze-Chung and Hsu (1987b) modified the SM medium by adding the antibiotics cephalexin and nitrofurantoin (SMA medium) and found this medium optimal for isolation of *X. c.* pv. *campestris*, compared to BSCAA, NSCA, NSCAA (see below) and SM. Schaad and Donaldson (1981), Randhawa and Schaad (1984) and Tze-Chung and Hsu (1987a) isolated antagonistic bacteria (*Bacillus* spp., fluorescent *Pseudomonas* spp.) from plates which bacteria were found to inhibit growth of *X. c.* pv. *campestris*. Because of a zero tolerance for black rot and the natural variation in the bacterial flora of crucifer seeds, Randhawa and Schaad (1984) suggested using NSCA (nutrient starch cycloheximide agar), NSCAA (NSCA with the addition of the antibiotics nitrofurantoin and vancomycin) as well as BSCAA (basal starch cycloheximide agar with the addition of the antibiotics nitrofurantoin and

Table 2. A summary of plating assays used for detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds.

Method	Sample treatment	Medium[1]	References
Direct plating of seeds	Seeds are disinfected (sodium hypochlorite) and directly plated on media	NSCA, SX	Schaad and Kendrick (1975), Schaad and Donaldson (1980)
Plating of liquid	A. Ethanol- and hypochlorite- disinfected seeds are soaked for 24 h in water; the suspension water is plated	Beef-peptone agar with starch	Lundsgaard (1973)
	B. Seeds are vigorously shaken for 1-5 min in saline (often with additives such as Tween 20, benomyl or chlorothalonil); seed washings are plated	BSCAA, FS, NSCA, NSCAA	Schaad and Donaldson (1980), Schaad (1982b, 1983a, 1983b), Randhawa and Schaad (1984), Schaad (1989)
	C. Seeds are vigorously shaken for 1 min in phosphate buffered saline with Tween; the wash solution is plated	BSCAA, NSCA, SM, SMA, SX	Tze-Chung and Hsu (1987a, 1987b)
	D. Seeds are shaken for 2 h in saline (with Tween 20 and benomyl); the wash solution is plated	CS20ABN, FS, NSCA, NSCAA	Chang et al. (1990, 1991)

[1]Some important components of these media are given below. BSCAA: basal starch cycloheximide agar with nitrofurantoin, vancomycin and methyl green, CS20ABN: medium with starch, bacitracin, neomycin and cycloheximide, FS: medium with starch, trimethoprim, cephalexin, cycloheximide, methyl green (and gentamicin), NSCA: Nutrient starch cycloheximide agar, NSCAA: NSCA with nitrofurantoin and vancomycin, SM: starch-methionine agar, with methyl violet 2B, methyl green, trace element solution, triphenyltetrazolium chloride and cycloheximide; SMA: SM-agar with cephalexin and nitrofurantoin; SX: selective medium with starch, methyl violet 2B, methyl green and cycloheximide.

vancomycin) to assay seeds for *X. c.* pv *campestris*. Yuen et al. (1987) used FS medium, developed by D. Fieldhouse and M. Sasser (unpublished), for isolation of *X. c.* pv. *campestris* from black rot lesion collected from the field. Schaad (1989) indicated that this medium, with the addition of gentamicin, could also be used to isolate *X. c.* pv. *campestris* from seeds. In a comparative test organized within the framework of the International Seed Testing Association (ISTA), this medium proved to be useful when longer extraction times were used (ISTA, unpublished). Chang et al. (1990, 1991) reported a new selective medium CS20ABN. This medium was developed because the extraction time was extended from 3 min to 2 h, which resulted in growth of numerous saprophytes. CS20ABN was compared to NSCA, NSCAA, and FS medium. Interference of saprophytic bacteria was less and recovery of *X. c.* pv. *campestris* was higher on CS20ABN than on the other media. Colony size of *X. c.* pv. *campestris* on CS20ABN was two- to threefold or three- to sevenfold larger than that on NSCA and NSCAA or FS, respectively. The percentage of pathogenic isolates was highest on NSCAA and lowest on CS20ABN. Of the 129 seed lots recorded positive, 69 were positive on NSCA, 55 positive on NSCAA and 121 were positive on CS20ABN. Eight samples were positive on NSCA and NSCAA, but negative on CS20ABN. Fifty two seed lots were positive on CS20ABN and negative on NSCA/NSCAA. The total number of negative seed lots was not stated.

From the literature cited it is clear that plating assays are used extensively. Despite the fact that more selective media have been developed, the success of isolation may still be variable, e.g. due to interference of saprophytes with the isolation assays, e.g. during the extraction. Recognition of suspected colonies may also be difficult because of the variable colony sizes on the media (A.A.J.M. Franken, unpublished) due to differences in EPS (extracellular polysaccharide) content and production (Cadmus et al., 1976; Kamoun and Kado, 1990; Kidby et al., 1977; Maiko, 1990). The differences in EPS production may be a result of a reaction to an (unknown) environmental stimulus and may influence the sensitivity of *X. c.* pv. *campestris* to antibiotics and salts (Osbourn et al.. 1990). This could also change the recovery of the pathogen on the media and the virulence of the bacterium, although the role of EPS in the pathogenicity of *X. c.* pv. *campestris* is not yet

fully understood (Coplin and Cook, 1990).

In all cases additional tests are necessary to finally confirm the identity of a suspected isolate.

Confirmation of suspected colonies from plating-assays

The most commonly used method for presumptive identification of suspected colonies from plating media is to use YDC (yeast-dextrose-carbonate) medium (Wilson et al., 1967; Schaad, 1989), followed by identification of typical colonies in immunofluorescence microscopy (Schaad, 1978). *Xanthomonas* colonies are yellow and mucoid on the YDC medium; the colony morphology being very typical. However, other tests may also be applicable for presumptive identification of *Xanthomonas* colonies such as reactions in ELISA (enzyme-linked immunosorbent assay) using monoclonal antibodies (Alvarez et al., 1985; Yuen et al., 1987) and polyclonal antibodies (Alvarez and Lou, 1985), reactions with phages (Bergamin and Kimati, 1981; Liew and Alvarez, 1981), fatty acid profiles (Stead, 1990), viscosity of bacterial suspensions (Pierce et al., 1990), sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of membrane proteins of *X. c.* pv. *campestris* (Thaveechai and Schaad, 1986a; Laakso et al., 1990), lipopolysaccharide patterns (Laakso et al., 1990), RFLP (restriction fragment length polymorphism) patterns of plasmids and possession of plasmids (Lazo and Gabriel, 1987), and RFLP patterns of genomic DNA (Lazo et al., 1987). The use of electrophoretic enzyme patterns for identifying taxon-specific patterns has also been suggested (El-Sharkawy and Huisingh, 1971). Identification of colonies belonging to the genus *Xanthomonas* may also be possible by isolation and characterization of the unique '*Xanthomonas*-carotenoid' (Starr and Stephens, 1964). Since the techniques mentioned generally cannot identify all strains on the pathovar level, a pathogenicity test is often needed for a check on production of specific symptoms. For other seed-borne bacteria such as *Pseudomonas syringae* pv. *phaseolicola* in bean seed, a pathogenicity test will generally give a definitive confirmation of the identity of a suspected isolate (Van Vuurde and Van den Bovenkamp, 1987). Because of their importance in seed testing, pathogenicity tests for *X. c.* pv. *campestris* will be discussed here in more detail.

Schaad (1982b) described a pathogenicity test as a part of a seed health testing method. In this test bacterial suspensions are injected in the stem of 4 to 6-week-old cabbage plants. Symptoms are vein blackening, followed by a slight wilting and necrosis of a part of the leaf. This test generally gives reliable results (A.A.J.M. Franken, unpublished). Hunter et al. (1987) evaluated seedlings for resistance by spraying a suspension of *X. c.* pv. *campestris* onto leaves with guttation droplets in a greenhouse mist chamber. Shaw and Kado (1988) compared several widely used inoculation techniques and concluded that *X. c.* pv. *campestris* was able to cause black rot quickly and at virtually all sites when inoculations were made at the hydathodes or wounds of intact plants. By this technique many other bacteria such as *Arthrobacter luteus, Erwinia carotovora* subsp. *carotovora, E. herbicola, Pseudomonas fluorescens, P. putida, P. viridiflava, Serratia marcescens* and *X. c.* pv. *vitians* failed to cause black rot. Robeson et al. (1989) and Bretschneider et al. (1989) introduced the inoculum via guttation droplets on the leaf margin, thereby avoiding mechanical injury to the plant and the possibility of producing artifacts. In all pathogenicity tests it should be taken into account that, depending on the environmental conditions, different symptoms may appear. Cook et al. (1952) described various symptoms at different temperatures: the characteristic marginal V-shaped regions predominated at higher temperatures (20, 24 and 28 °C), subsequently followed by the appearance of more systemic lesions, predominating in more central areas of the leaves. At the lowest temperature (16 °C) only marginal lesions were observed. A third symptom type, a chlorotic spotting not previously associated with black rot often appeared soon after inoculation and was attributed to a toxic substance. Machmud and Black (1985) found that xanthomonas leaf spot caused by the closely related *X. c.* pv. *amoraciae* differs from black rot of crucifers caused by *X. c.* pv. *campestris* in that no chlorosis is associated with the lesions. *X. c.* pv. *amoraciae* invades the stomata but fails to move systemically and the disease develops at temperatures well below those favourable for black rot. Inoculation of field grown cabbage, cauliflower, and broccoli with *X. c.* pv. *amoraciae* showed that these crops are susceptible and that disease severity varies among cabbage cultivars. Unfortunately, strains of some pathovars can be pathogenic on host species in different plant families (Leyns et al., 1984). For example, *X. c.* pv. *amoraciae*

occurs on leaves of *Radicula amoracia* and is a weak pathogen of cabbage and cauliflower; *X. c.* pv. *aberrans* is also able to infect crucifers and has high virulence in cauliflower (Hayward and Waterston, 1965).

Nonpathogenic or weakly virulent strains of *X. c.* pv. *campestris*, strains of *X. c.* pv. *campestris* which give aberrant symptoms as well as *X. c.* pv. *amoraciae* may be found in crucifer seed (A.A.J.M. Franken and G.W. van den Bovenkamp, unpublished; Yuen and Alvarez, 1985; Machmud and Black, 1985).

Therefore it is hard to precisely define the differences in symptoms caused by e.g. *X. c.* pv. *amoraciae* and *X. c.* pv. *campestris*. Extra complicating factors are the effects of temperature, humidity and seed treatments, variation in virulence among strains of *X. c.* pv. *campestris*, specific interactions with different *Brassica* species and cultivars, and the type of infection (hydathodal or stomatal).

Some of the problems are specifically related to the nomenclature and taxonomy of pathovars of *X. campestris* and are in part due to the convention that pathovars are named after the plant from which they were first isolated and caused symptoms. Hildebrand et al. (1990) showed by DNA-DNA hybridization tests that pathogenicity towards members of the same host family is not a measure of the genomic relationship of xanthomonads. Vauterin et al. (1990) discussed the taxonomic problems of the genus *Xanthomonas* and especially of *X. campestris*. They also concluded that the host selectivity was not correlated with relationships as revealed by DNA-DNA hybridization experiments. Since more xanthomonads can be found on one plant species and in view of the aberrant symptom production of some isolates as mentioned above, it is clear that classifying isolates as 'pathovar', on the basis of symptom production on a test plant can be very complicated. Taxonomic studies have not yet been concluded, so there are still problems in naming isolates derived from seed lots, especially when some isolates differ from the general population in the seed lots.

Pathogenicity tests should be used for final proof of the identity of the isolate based on the appearance of 'specific' symptoms on cabbage or another host plant. However, they will not always give 100% proof of the identity of an isolate at the pathovar level. Therefore, pathogenicity testing needs therefore to be combined with tests such as

serology (ELISA, immunofluorescence microscopy) and other tests mentioned above. It remains questionable whether application of a combination of these tests will discriminate between closely related pathovars of *X. campestris* and *X. c.* pv. *campestris*.

Serology

Since serological techniques often play an important role in the detection or identification of *X. c.* pv. *campestris* from seed, special attention has been paid to this subject.

Schaad (1982a) discussed the main advantages and disadvantages of serological tests for detection and identification of seed-borne bacteria. The main advantages of serological tests are speed and low cost. Disadvantages are the risk of cross-reactions and the detection of dead cells.

Serological tests are used world-wide for seed-borne bacteria (Franken and Van Vuurde, 1990). Two aspects of serology are very important and receive much attention in the literature, i.e. the serological relationship between pathovars of *X. campestris* and the production and use of specific antisera.

Antigens used and serological relationships with other pathovars of Xanthomonas campestris

Elrod and Braun (1947a) used agglutination and absorption experiments with polyclonal antisera to study the serological relationship of several pathovars of *X. campestris*. *X. c.* pv. *campestris*- antisera cross-reacted with *X. c.* pv. *vesicatoria*, *X. c.* pv. *raphani* and with *X. c.* pv. *amoraciae*. They concluded that the ability to infect a given host was in certain instances not reflected in the measurable antigenic make-up of the organism. They (Elrod and Braun, 1947b) also found that when using antisera prepared against several pathovars of *X. campestris* the two strains in the *X. c.* pv. *campestris* group (*X. c.* pv. *campestris* and *X. c.* pv. *barbarae*) had a serologically intermediate position between strains in the *X. vascularum* group (e.g. *X. c.* pvs. *vesicatoria*, *incanae*, *amoraciae*) and the *X. phaseoli* group (e.g. *X. c.* pvs. *pelargonii*, *phaseoli*, *phaseoli* var. fuscans, *malvacearum*).

Volk (1966) investigated the composition of the lipopolysaccharides (LPS) from 20 *Xanthomonas* species, most of them now classified as pathovars of *X. campestris*. Qualitative analysis of the components of the polysaccharide portion of *Xanthomonas* LPS showed that *X. c.* pv. *campestris* differed from other species tested (e.g. *X. c.* pv. *phaseoli*, *X. c.* pv. *begoniae*, *X. c.* pv. *hyacinthi*; *X. c.* pvs. *amoraciae* and *vesicatoria* were not tested) by the release of galactose from the isolated LPS upon acid treatment. All LPS of *Xanthomonas* species released rhamnose and contained uronic acid, ketodeoxyoctonate, glucose and mannose. Considering the homology and differences in LPS between *Xanthomonas* spp. and pathovars this study suggests that the LPS might be of use to produce specific antisera.

Schaad (1978) prepared polyclonal antisera to ribosomes. All strains of *X. c.* pv. *campestris* reacted with these antisera, only strains of *X. c.* pv. *vesicatoria* cross-reacted with the antisera prepared against ribosomes of *X. c.* pv. *campestris* in direct and indirect immunofluorescence microscopy (IF). Thaveechai and Schaad (1984) used four immunogens (formaldehyde fixed cells, glutaraldehyde fixed cells, trichloroacetic acid extracts and ribosomal extracts) to produce antisera. The pattern and number of precipitin bands in Ouchterlony double diffusion (ODD) were similar for the four immunogens. In IF few differences among immunogens were found. However, none of the immunogens, were specific enough in IF to differentiate *X. c.* pv. *campestris* from other xanthomonads. Cross-reactions with strains of *X. c.* pv. *vesicatoria* and *X. c.* pv. *translucens* were found in IF and ODD. Other bacteria failed to react.

Alvarez et al. (1985) produced monoclonal antibodies to living cells. By using a combination of four monoclonal antibodies (X1, X9, X13 and X17) in ELISA, strains of *X. c.* pv. *campestris* could be distinguished from other pathovars of *X. campestris* and could be grouped and subgrouped. The authors suggested that these monoclonals might be applied as markers for rapid identification of *X. c.* pv. *campestris* strains and for tracing strains in epidemiological studies of black rot of crucifers. Individual monoclonal antibodies cross-reacted with other pathovars of *X. campestris* (e.g. *X. c.* pv. *amoraciae*) and/or did not react with all strains of *X. c.* pv. *campestris*. No information was given on the specific antigens reacting with the monoclonal antibodies.

Thaveechai and Schaad (1986a) characterized LiCl-extracted membrane proteins of strains of *X. c.* pv. *campestris* from Thailand by serology and SDS-PAGE. SDS-PAGE-profiles of membrane proteins of *X. c.* pv. *campestris* were distinct from other bacteria. However, this was only valid for the 'typical' strains, producing yellowing and black veins in cabbage seedlings. The 'atypical' strains, that gave black lesions and no yellowing or black veins, showed less homology with the 'typical' strains and considerable homology with *X. c.* pv. *vesicatoria* and pv. *incanae*. Antisera were made to membrane proteins of seven strains. In ODD and IF, strains of *X. c.* pv. *vesicatoria*, *X. c.* pv. *incanae* cross-reacted. Results of this study demonstrated that membrane proteins contain an immunogen of high specificity at the subspecies level.

Thaveechai and Schaad (1986b) identified the specific antigenic moiety of membrane proteins as a heat-stable inner membrane peptide, which was thought to be a part of a complex molecule. Haaheim et al. (1989) produced monoclonal antibodies against the extracellular polysaccharide (EPS) xanthan from a strain of *X. c.* pv. *campestris*. All antibodies reacted with a range of different xanthans. Using immunoblotting techniques on nitrocellulose paper both a mixture of monoclonal antibodies, and also polyclonal ascitic fluid, could detect xanthan quantities of ca. 0.1 microgram. The antibodies were not tested in detail for cross-reactions with extracellular polysaccharides of other pathovars of *X. campestris*.

The studies cited above show that antisera produced against *X. c.* pv. *campestris* commonly cross-react with other pathovars such as *X. c.* pv. *amoraciae* and *X. c.* pv. *vesicatoria*. Typical antigens could be membrane proteins and the LPS of *X. c.* pv. *campestris*. No results have been reported by these groups with respect to application of antibodies in serological tests for indexing plant material.

Applications of serological tests

Other research workers have reported the application of serological tests for detection of *X. c.* pv. *campestris* in plant material or identification of suspected colonies (pure colonies). For instance, Lundsgaard (1973) used agglutination for identification of suspected colonies. Schaad and Donaldson (1980) applied IF with polyclonal antibodies

for detection of *X. c.* pv. *campestris* in seed washings, and reported that of 121 commercial crucifer seed lots tested six samples were positive by plating seed washings onto an agar medium (NSCA), two were positive by the serological test immunofluorescence microscopy (IF) and none were positive by plating seeds onto an agar medium. Comparison of IF assays with field incidence of black rot showed a low correlation of IF with disease in the field (Schaad, 1982a, 1983a, 1983b). Schaad (1983b) proposed that unless high correlations with field disease are established, serological assays such as IF for seedborne bacteria should not be used for certification programmes. However, IF was still suitable for identification of suspected colonies.

Alvarez and Lou (1985) developed a double-antibody sandwich ELISA (DAS-ELISA) to detect *X. c.* pv. *campestris* in leaf discs sampled from cabbage in the field. The polyclonal antisera were raised in sheep and goat and did not react with all strains of *X. c.* pv. *campestris*. They cross-reacted with strains of *X. c.* pv. *vesicatoria, amoraciae, and vitians*. DAS-ELISA was not as sensitive as IF, but was considered to be sufficiently sensitive to detect levels of bacteria associated with a variety of lesion types in leaves. As compared to isolation and pathogenicity tests DAS-ELISA gave a predictive value for a positive result of 92.6% and for a negative result of 91.1% at a disease incidence (regarded by these authors to be identical to 'prevalence' of a disease) of 55.5%.

Yuen et al. (1987) used an indirect ELISA with four monoclonal antibodies (X3, X9, X11 and X20, as reported by Alvarez et al. (1985) to detect and differentiate strains of *X. c.* pv. *campestris* isolated on FS medium from black rot lesions collected from the field. They stated that the antibody reactivity patterns of strains of *X. c.* pv. *campestris*, introduced in the field for epidemiological studies, were stable in serial passage through plant and media. Alvarez and Benedict (1990) discussed the application of the monoclonals developed by Alvarez et al. (1985). A typical reaction with two or more of the four monoclonal antibodies (X9, X13, X17, X21) was generally indicative of a typical, virulent black rot organism. When only one out of four monoclonal antibodies reacted, the strain was generally less virulent or produced atypical symptoms that developed slowly. Avirulent xanthomonads recovered from crucifer leaf surfaces and crucifer seeds reacted with X11 and/or X1. Testing over a thousand *X. c.* pv. *campestris*

strains, they found repeating patterns from strains isolated from widely separated geographical origin.

As shown in the literature on serology for *X. c.* pv. *campestris*, several serological techniques, such as ELISA, ODD, agglutination and IF were used for studying pure cultures of the pathogen. For this purpose the sensitivity of the technique is of minor importance since high cell concentrations can be used. For direct detection of *X. c.* pv. *campestris* in plant material only ELISA and IF have so far been used and, only IF has been used for seed testing (Schaad and Donaldson, 1980). IF has a relatively high sensitivity (Franken and Van Vuurde, 1990) and is therefore suited to detect low levels of infection amongst high numbers of saprophytes.

Concluding remarks

To determine the 'health status' of a seed lot, the seed should be tested for presence of the pathogen. Most commonly, seeds are washed (shaken or soaked) in a solution and washings are plated on media, such as BSCAA, CS20ABN, FS, NSCA, NSCAA, SM, and SMA. Suspected colonies on these media are generally confirmed on YDC-medium, with serological techniques (IF, agglutination, ELISA) and pathogenicity tests. In a few instances, serological techniques have been used to directly detect *X. c.* pv. *campestris* in plant material. To date IF is the only serological technique used to detect the black rot pathogen in seed washings (Schaad and Donaldson, 1980).

Acknowledgements

Thanks are due to prof. dr ir J. Dekker and dr C. Langerak for critically reading the manuscript.

References

Alvarez, A.M. & Benedict, A.A., 1990. Relationships among phytopathogenic bacteria distinguished with monoclonal antibodies. Proceedings of the 7th International Conference on Plant Pathogenic Bacteria, Hungary, pp. 859-863 (ed: Z. Klement). Akadémiai Kiadó, Budapest.

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

Alvarez, A.M., Benedict, A.A. & Mizumoto, C.Y., 1985. Identification of xanthomonads and grouping of strains of *Xanthomonas campestris* pv. *campestris* with monoclonal antibodies. Phytopathology 75: 722-728.

Aveling, T.A.S. & Robbertse, P.J., 1990. Evaluation of antibiotics against *Xanthomonas campestris* causing black rot of *Brassica*. Phytophylactica 22: 229-231.

Bergamin Filho, A. & Kimati, H., 1981. Studies on a bacteriophage isolated from *Xanthomonas campestris*. III. Detection of *Xanthomonas campestris* in cabbage by means of phages. Summa Phytopathologica 7: 26-33 (Summary in English).

Bhardwaj, L.N., Shyam, K.R. & Thakur, P.D., 1987. Effect of seed and soil treatments on the incidence of damping-off and black rot in cauliflower seedlings. Pesticides 21: 13.

Bretschneider, K.E., Gonella, M.P. & Robeson, D.J., 1989. A comparative light and electron microscopical study of compatible and incompatible interactions between *Xanthomonas campestris* pv. *campestris* and cabbage (*Brassica oleracea*). Physiological and Molecular Plant Pathology 34: 285-297.

Bucha, J., 1977. Studies on some bacterial isolates from *Brassica oleracea* L. var. *capitata* L. seedlings which were obtained in the Srinivasan et al. test for detection of *Xanthomonas campestris* (Pammel) Dowson. Report Danish Government Institute of Seed Pathology for Developing Countries, Copenhagen, Denmark.

Cadmus, M.C., Rogovin, S.P., Burton, K.A., Pittsley, J.E., Knutson, C.A. & Jeanes, A., 1976. Colonial variation in *Xanthomonas campestris* NRRL B-1459 and characterization of the polysaccharide from a variant strain. Canadian Journal of

Microbiology 22: 942-948.

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

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

Chun, W.W.C. & 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.

Clayton, E.E., 1924. Control of black-rot and black-leg of cruciferous crops by seed and seed bed treatments. Phytopathology 14: 24-25 (Abstract).

Clayton, E.E., 1925. Second progress report of black rot (*Pseudomonas campestris*) investigations on Long Island: seed infection and seasonal development. Phytopathology 15: 48-49 (Abstract).

Cook, A.A., Walker, J.C. & Larson, R.H., 1952. Studies on the disease cycle of black rot of crucifers. Phytopathology 42: 162-167.

Coplin, D.L. & Cook, D., 1990. Molecular genetics of extracellular polysaccharide biosynthesis in vascular phytopathogenic bacteria. Molecular Plant-Microbe Interactions 3: 271-279.

El-Sharkawy, T.A. & Huisingh, D., 1971. Differentiation among *Xanthomonas* species by polyacrylamide gel electrophoresis of soluble proteins. Journal of General Microbiology 68: 155-165.

Elrod, R.P. & Braun, A.C., 1947a. Serological studies of the genus *Xanthomonas*. I. Cross-agglutination relationships. Journal of Bacteriology 53: 509-518.

Elrod, R.P. & Braun, A.C., 1947b. Serological studies of the genus *Xanthomonas*. III. The *Xanthomonas vascularum* and *Xanthomonas phaseoli* groups; the intermediate position of *Xanthomonas campestris*. Journal of Bacteriology 54: 349-357.

Franken, A.A.J.M. & Van Vuurde, J.W.L., 1990. Problems and new approaches in the

use of serology for seed-borne bacteria. Seed Science and Technology 18: 415-426.

Haaheim, L.R., Kleppe, G. & Sutherland, I.W., 1989. Monoclonal antibodies reacting with the exopolysaccharide xanthan from *Xanthomonas campestris*. Journal of General Microbiology 135: 605-612.

Hankin, L. & Sands, D.C., 1977. Microwave treatment of tobacco seed to eliminate bacteria on the seed surface. Phytopathology 67: 794-795.

Harman, G.E., Norton, J.M., Stasz, T.E. & Humaydan, H.S., 1987. Nyolate seed treatment of *Brassica* spp. to eradicate or reduce black rot caused by *Xanthomonas campestris* pv. *campestris*. Plant Disease 71: 27-30.

Hayward, A.C. & Waterston, J.M., 1965. C.M.I. Description of pathogenic fungi and bacteria no. 47. *Xanthomonas campestris*.

Hildebrand, D.C., Palleroni, N.J. & Schroth, M.N., 1990. Deoxyribonucleic acid relatedness of 24 xanthomonad strains representing 23 *Xanthomonas campestris* pathovars and *Xanthomonas fragariae*. Journal of Applied Bacteriology 68: 263-269.

Huang, T.C. & Lee, H.L., 1988. Hot acidified zinc sulfate as seed soaking agent for the control of crucifer black rot. Plant protection Bulletin Taiwan 30: 245-258.

Humaydan, H.S., Harman, G.E., Nedrow, B.L. & DiNito, L.V., 1980. Eradication of *Xanthomonas campestris*, the causal agent of black rot, from *Brassica* seeds with antibiotics and sodium hypochlorite. Phytopathology 70: 127-131.

Hunter, J.E., Dickson, M.H. & Ludwig, J.W., 1987. Source of resistance to black rot expressed in young seedlings and adult plants. Plant Disease 71: 263-266.

Kamoun, S. & Kado, C.I., 1990. Phenotypic switching affecting chemotaxis, xanthan production, and virulence in *Xanthomonas campestris*. Applied and Environmental Microbiology 56: 3855-3860.

Kidby, D., Sandford, P., Herman, A. & Cadmus, M., 1977. Maintenance procedures for the curtailment of genetic instability: *Xanthomonas campestris* NRRL B-1459. Applied and Environmental Microbiology 33: 840-845.

Kishun, R., 1984. Seed treatment for control of cabbage black rot. Journal of Turkish Phytopathology 13: 81-86.

Klisiewicz, J.M. & Pound, G.S., 1961. Studies on control of black rot of crucifers by

treating seeds with antibiotics. Phytopathology 51: 495-500.

Laakso, T., Ojanen, T., Helander, I.M., Karjalainen, R., Korhonen, T.K. & Haahtela, K., 1990. Comparison of outer membrane proteins and lipopolysaccharides of *Xanthomonas campestris* pathovars. Proceedings of the 7th International Conference on Plant Pathogenic Bacteria, Hungary, pp. 149-154 (ed: Z. Klement). Akadémiai Kiadó, Budapest.

Lazo, G.R. & Gabriel, D.W., 1987. Conservation of plasmid DNA sequences and pathovar identification of strains of *Xanthomonas campestris*. Phytopathology 77: 448-453.

Lazo, G.R., Roffey, R. & Gabriel, D.W., 1987. Pathovars of *Xanthomonas campestris* are distinguishable by Restriction Fragment-Length Polymorphism. International Journal of Systematic Bacteriology 37: 214-221.

Liew, K.W. & Alvarez, A.M., 1981. Phage typing and lysotype distribution of *Xanthomonas campestris*. Phytopathology 71: 274-276.

Leyns, F, De Cleene, M., Swings, J. & De Ley, J., 1984. The host range of the genus *Xanthomonas*. The Botanical Review 50: 308-356.

Lin, C.Y., 1981. Studies on black rot of cruciferous crops caused by *Xanthomonas campestris* pv. *campestris* in Taiwan. Plant Protection Bulletin Taiwan 23: 157-167.

Lundsgaard, T., 1973. A method for detection of *Xanthomonas campestris* (Pammel) Dowson in *Brassica* seeds. In: 24. Årsberetning vedrørende Frøpatologisk Kontrol, 1. juni 1971-31. maj 1972. Ed: Henning Andersen (Summary in English).

Machmud, M. & Black, L.L., 1985. A *Xanthomonas* leaf spot of crucifers caused by *Xanthomonas campestris* pv. *amoraciae*. Contributions of the Central Research Institute for Food Crops Bogor 74: 24 p.

Maiko, I.I., 1990. Exopolysaccharides of *Xanthomonas* and their properties. Proceedings of the 7th International Conference on Plant Pathogenic Bacteria, Hungary, pp. 155-159 (ed: Z. Klement). Akadémiai Kiadó, Budapest.

McKeen, W.E., 1981. Black rot of rutabaga in Ontario and its control. Canadian Journal of Plant Pathology 3: 244-246.

Navaratnam, S.J., Shuttleworth, D. & Wallace, D., 1980. The effect of aerated steam on

six seed-borne pathogens. Australian Journal of Experimental Agriculture and Animal Husbandry 20: 97-101.

Osbourn, A.E., Clarke, B.R., Stevens, B.J.H. & Daniels, M.J., 1990. Use of oligonucleotide probes to identify members of two-component regulatory systems in *Xanthomonas campestris* pathovar *campestris*. Mol Gen Genet 222: 145-151.

Pierce, L., Schroth, M.N. & McCain, A.H., 1990. Viscosity test for preliminary identification of strains of *Xanthomonas campestris*. Plant Disease 74: 646-647.

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

Robeson, D.J., Bretschneider, K.E. & Gonella, M.P., 1989. A hydathode inoculation technique for the simulation of natural black rot infection of cabbage by *Xanthomonas campestris* pv. *campestris*. Annals of Applied Biology 115: 455-459.

Schaad, N.W., 1976. Control of black rot of cabbage. Research Bulletin 187. Georgia Experiment Station, Georgia, USA.

Schaad, N.W., 1978. Use of direct and indirect immunofluorescence tests for identification of *Xanthomonas campestris*. Phytopathology 68: 249-252.

Schaad, N.W., 1982a. Detection of seedborne bacterial plant pathogens. Plant Disease 66: 885-890.

Schaad, N.W., 1982b. *Xanthomonas campestris* pv. *campestris*. Working sheet no. 50. ISTA Handbook on Seed Health Testing. ISTA, Zürich, Switzerland.

Schaad, N.W., 1983a. Detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds. Seed Science and Technology 11: 573-578.

Schaad, N.W., 1983b. Correlation of laboratory assays for seedborne bacteria with disease development. Seed Science and Technology 11: 877-883.

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

Schaad, N.W. & Donaldson, R.C., 1980. Comparison of two methods for detection of *Xanthomonas campestris* in infected crucifer seeds. Seed Science and Technology 8:

383-391.

Schaad, N.W. & Donaldson, R.C., 1981. Bacteria of crucifer seeds antagonistic to *Xanthomonas campestris*. Phytopathology 71: 902 (Abstract).

Schaad, N.W. & Kendrick, R., 1975. A qualitative method for detecting *Xanthomonas campestris* in crucifer seed. Phytopathology 65: 1034-1036.

Schaad, N.W. & Thaveechai, N., 1983. Black rot of crucifers in Thailand. Plant Disease 67: 1231-1234.

Schaad, N.W. & White, W.C., 1974. A selective medium for soil isolation and enumeration of *Xanthomonas campestris*. Phytopathology 64: 876-880.

Schaad, N.W., Gabrielson, R.L. & Mulanax, M.W., 1980. Hot acidified cupric acetate soaks for eradication of *Xanthomonas campestris* from crucifer seeds. Applied and Environmental Microbiology 39: 803-807.

Schultz, T., Gabrielson, R.L. & Olson, S., 1986. Control of *Xanthomonas campestris* pv. *campestris* in crucifer seed with slurry treatments of calcium hypochlorite. Plant Disease 70: 1027-1030.

Shackleton, D.A., 1962. A method for the detection of *Xanthomonas campestris* (Pammel, 1895) Dowson, 1939, in *Brassica* seed. Nature 193: 78.

Shah, A., Srivastava, K.K., Roy, A.J. & Bora, S.S., 1985. Control of black rot disease of cauliflower by seed treatment. Progressive Horticulture 17: 72-74.

Sharma, S.L., 1981. Control of black rot of cauliflower by hot water seed treatment and field sprays with streptocycline. Indian Journal of Mycology and Plant Pathology 11: 17-20.

Shaw, J.J. & Kado, C.I., 1988. Whole plant wound inoculation for consistent reproduction of black rot in crucifers. Phytopathology 78: 981-986.

Shekhawat, P.S., Jain, M.L. & Chakravarti, B.P., 1982. Detection and seed transmission of *Xanthomonas campestris* pv. *campestris* causing black rot of cabbage and cauliflower and its control by seed treatment. Indian Phytopathology 35: 442-447.

Smith, E.F., 1920. An introduction to bacterial diseases of plants. W.B. Saunders Company, USA. pp. 688.

Srinivasan, M.C., Neergaard, P. & Mathur, S.B., 1973. A technique for detection of

Xanthomonas campestris in routine seed health testing of crucifers. Seed Science and Technology 1: 853-859.

Starr, M.P. & Stephens, W.L., 1964. Pigmentation and taxonomy of the genus *Xanthomonas*. Journal of Bacteriology 87: 293-302.

Stead, D.E., 1990. Evaluation of cellular fatty acid profiles for rapid identification of plant pathogenic bacteria. Proceedings of the 7th International Conference on Plant Pathogenic Bacteria, Hungary, pp. 483-490 (ed: Z. Klement). Akadémiai Kiadó, Budapest.

Thaveechai, N. & Schaad, N.W., 1984. Comparison of different immunogen preparations for serological identification of *Xanthomonas campestris* pv. *campestris*. Phytopathology 74: 1065-1070.

Thaveechai, N. & Schaad, N.W., 1986a. Serological and electrophoretic analysis of a membrane protein extract of *Xanthomonas campestris* pv. *campestris* from Thailand. Phytopathology 76: 139-147.

Thaveechai, N. & Schaad, N.W., 1986b. Immunochemical characterization of a subspecies-specific antigenic determinant of a membrane protein extract of *Xanthomonas campestris* pv. *campestris*. Phytopathology 76: 148-153.

Tze-Chung, H. & Hsu, S.T., 1987a. Efficiency of various selective media for detection of *Xanthomonas campestris* pv. *campestris* in Taiwan. Plant Protection Bulletin Taiwan 29: 203-215.

Tze-Chung, H. & Hsu, S.T., 1987b. Detection techniques for *Xanthomonas campestris* pv. *campestris* in crucifer seeds and soils in Taiwan by isolation with a selective medium. Plant Protection Bulletin Taiwan 29: 217-231.

Van Vuurde, J.W.L. & Van den Bovenkamp, G.W., 1987. ISTA Handbook on seed health testing. Working sheet no 65, *Pseudomonas syringae* pv. *phaseolicola*, pp. 8.

Vauterin, L., Swings, J., Kersters, K., Gillis, M., Mew, T.W., Schroth, M.N., Palleroni, N.J., Hildebrand, D.C., Stead, D.E., Civerolo, E.L., Hayward, A.C., Maraite, H., Stall, R.E., Vidaver, A.K. & Bradbury, J.F., 1990. Towards an improved taxonomy of *Xanthomonas*. International Journal of Systematic Bacteriology: 312-316.

Volk, W.A., 1966. Cell wall lipopolysaccharides from *Xanthomonas* species. Journal of

Bacteriology 91: 39-42.

Walker, J.C., 1952. Diseases of vegetable crops. McGraw-Hill Book Company, Inc., New York, pp. 128-131.

Walker, J.C. & Tisdale, W.B., 1920. Observations on seed transmission of the cabbage black rot organism. Phytopathology 10: 175-177.

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

Wilson, E.E., Zeitoun, F.M. & Fredrickson, D.L., 1967. Bacterial phloem canker, a new disease of persian Walnut trees. Phytopathology 57: 618-621.

Yuen, G.Y.K. & Alvarez, A.M., 1985. Aberrant symptoms on cabbage caused by strains of *Xanthomonas campestris*. Phytopathology 75: 1382 (Abstract).

Yuen, G.Y., Alvarez, A.M., Benedict, A.A. & Trotter, K.J., 1987. Use of monoclonal antibodies to monitor the dissemination of *Xanthomonas campestris* pv. *campestris*. Phytopathology 77: 366-370.

Chapter 3. Evaluation of a plating assay for *Xanthomonas campestris* pv. *campestris*

Evaluation of a plating assay for *Xanthomonas campestris* pv. *campestris*

Abstract

A plating assay for detecting *Xanthomonas campestris* pv. *campestris* in crucifer seeds was evaluated. The results showed no significant effect of a centrifugation step prior to plating compared to an assay without a centrifugation step with respect to logarithms of colony-forming units (log cfu) of *X. c.* pv. *campestris* per ml. The addition of Tween 20, Benlate (benomyl) and Daconil (chlorothalonil) to saline (0.85% NaCl) generally resulted in less log cfu of *X. c.* pv. *campestris* per ml. The extraction methods 2.5 hours shaking at room temperature (21 $\pm$ 2 °C) and 1.5 hours soaking in a refrigerator (4 - 6 °C) gave generally more log cfu of *X. c.* pv. *campestris* per ml than 5 minutes shaking the seeds at room temperature. However, 2.5 hours shaking or 1.5 hours soaking of seed lots did not result in finding more positive (=infected) seed lots than 5 minutes shaking. No significant differences were found between the plating media NSCA and NSCAA for any of the extraction methods with respect to log cfu of *X. c.* pv. *campestris*, the number of positive seed lots, or the number of positive subsamples per seed lot. The FS medium was compared to NSCA and NSCAA in one experiment using 5 minutes shaking the seed lots in saline at room temperature. No significant differences were found between NSCA, NSCAA and FS medium with respect to the number of positive seed lots. Also no differences were found between testing seed lots in five subsamples of 2000 seeds or one sample of 10 000 seeds with regard to the log cfu of *X. c.* pv. *campestris* per ml and the number of positive seed lots. A general scheme for routine testing in the Netherlands is presented.

Introduction

Xanthomonas campestris pv. *campestris* is the causal organism of black rot of crucifers. The bacterium is seed-borne and infected seed is an important source of inoculum (Schaad, Sitterly and Humaydan, 1980). Several plating methods and media have been published for detecting *X. c.* pv. *campestris* in crucifer seeds and were summarized by Schaad (1989). Plating of seed washings is considered to be the most reliable and efficient method for routine detection of *X. c.* pv. *campestris* (Schaad, 1989). Five minutes washings of seed lots is generally described as a standard procedure (e.g. Schaad, 1982). However, it is known that longer washings can increase the number of colony-forming units (cfu) of *X. c.* pv. *campestris* (ISTA, 1988; Schaad, 1989; Chang, Donaldson, Crowley and Pinnow, 1989a). Agar media for isolation of *X. c.* pv. *campestris* from plant tissues, e.g. seeds, or soil are: SX-agar (Schaad and White, 1974), SM-agar (Chun and Alvarez, 1983), NSCA (Schaad and Kendrick, 1975), NSCAA (Randhawa and Schaad, 1984), BSCAA (Randhawa and Schaad, 1984), FS (Fieldhouse and Sasser, unpublished; Schaad, 1989), CS20ABN medium (Chang, Donaldson, Crowley and Pinnow, 1989a; Chang, Donaldson, Crowley and Pinnow, 1989b). The latter medium was reported during this study. NSCA, NSCAA, BSCAA, FS and/or CS20ABN are now generally used at various laboratories for detecting *X. c.* pv. *campestris* particularly in seeds. Identification of suspected colonies is normally done by transferring colonies to YDC medium, sometimes followed by immunofluorescence staining. Finally, suspected colonies are confirmed by pathogenicity testing (Schaad, 1989).

This study was carried out to evaluate and specify parts of the plating assay for *X. c.* pv. *campestris*, based on the report given by Schaad (1982). Special attention was paid to the suitability of the method for routine use.

Materials and methods

Extraction of Xanthomonas campestris pv. campestris from crucifer seeds

All seed lots used were naturally contaminated. Five subsamples of 2000 seeds were tested per seed lot (analogous to testing five subsamples of 1000 bean seeds for *Pseudomonas syringae* pv. *phaseolicola*, Van Vuurde, Van den Bovenkamp and Birnbaum (1983)), unless otherwise stated. In initial experiments with seed lots B197 and B189 20 ml of sterile saline (0.85% NaCl, (w/v)), with 20 µl Tween 20, 5 mg Benlate (75% active ingredient) and 100 µl of a 1:10 diluted Daconil suspension (Daconil contains in its undiluted form 50% chlorothalonil) was added to each subsample of 2000 seeds. In subsequent experiments no Tween 20, Benlate or Daconil was added to saline. When 10 000 seeds were tested as a whole, 100 ml saline was added to each sample of 10 000 seeds. Seeds were either vigorously shaken during 5 minutes or 2.5 hours at room temperature (21 ± 2 °C), were soaked for 1.5 hours in a refrigerator (4 - 6 °C), or were extracted under vacuum. For the extraction under vacuum seeds were put in a desiccator. Air was removed with a vacuum pressure pump for 5 minutes. After 5 minutes the desiccator was opened again for air to enter. Seeds were vigorously shaken for a few seconds. All extraction methods were tested on different subsamples of a seed lot, except for 5 minutes and 2.5 hours shaking which were tested on the same subsamples of a seed lot.

The effect of centrifugation was investigated for 5 minutes shaking of seeds only (testing 5 x 2000 seeds). Washings of each subsample of 2000 seeds were filtered through a cheese cloth and centrifuged for 10 minutes at 10 000 g. Each pellet was resuspended in 400 µl of sterile saline. Thus the seed washings were x50 concentrated. Serial ten-fold dilutions were made from this suspension (a 10^{-1}, 10^{-2} and 10^{-3} dilution). Other subsamples from the same seed lots were not centrifuged and serial dilutions were made directly from the seed washings (a x2, x20 and x200 dilution). The latter method (no centrifugation) was used in all other experiments.

Isolation of Xanthomonas campestris pv. campestris

Fifty microliter of the seed washing of each subsample was plated in three ten-fold dilutions in duplicate on NSCA and NSCAA. In one experiment using 5 minutes shaking the FS medium was included. NSCA, NSCAA and FS were modified by adding soluble starch at a concentration of 25 g/l instead of 10 g/l (NSCA; Schaad and Kendrick, 1975) or 15 g/l (NSCAA and FS; Schaad, 1989). All plates were placed in the refrigerator at least five days prior to use. All plates were used between five and fourteen days after preparation. Inoculated plates were incubated at 25 °C for three days. Reference strains IPO 634 (isolated from seeds of *Brassica oleracea* var. *gemmifera* in the Netherlands) and IPO 671 (donated by dr. N.W. Schaad as B24) were plated as controls.

Identification of suspected colonies on YDC

A minimum of five suspected colonies (if present) per subsample were transferred from NSCA, NSCAA or FS to YDC. Minimally two saprophytes per subsample were tested on YDC. YDC-plates were incubated for two days at 30 °C. After identification on YDC, minimally two suspected colonies (on YDC) per subsample were confirmed in the pathogenicity test. At least two saprophytes per subsample were confirmed in the pathogenicity tests. Reference strains IPO 634 and IPO 671 were used as controls.

Pathogenicity testing

Schaad's pathogenicity test (Schaad, 1982), was used as a reference. For all pathogenicity tests susceptible varieties of *Brassica oleracea* were used. Cultures were grown for 24-48 hours at 27 °C on a growth factor medium, containing per liter: 0.4 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.5 g $NH_4H_2PO_4$, 0.01 g $FeCl_3$, 1.0 g glucose, 3.0 g yeast extract and 15.0 g Bacto-agar. Light milky suspensions (approximately 10^7 cells/ml) were made in distilled water and inoculations took place immediately after preparation of the suspensions.

Processing of the data

Each plate with less than 250 colonies was counted. All data were converted to colony-

forming units (cfu) per ml of non-centrifuged seed washings. For statistical processing, logarithms to base 10 (log) of the cfu of *X. c.* pv. *campestris* and of the total cfu were calculated. To include all zero readings of the plates, 1 cfu was added to all cfu. Analysis of variance (ANOVA) was done by using the program Genstat 5 (Rothamsted Experimental Station). Means, variances and standard errors of differences of means were computed from subsamples, replicate plates and the x20 dilution, unless otherwise stated. For statistical reasons, values of 5 minutes and 2.5 hours shaking were not both compared with other extraction methods in one ANOVA. Therefore, standard errors of differences of means are given for each relevant comparison of treatments. Values are not adjusted for missing values. Nonparametric tests were done as described in Siegel (1956). All tests were done at 95% probability.

Results

Centrifugation of seed washings

To investigate the necessity of a centrifugation step prior to plating, two seed lots were tested by three laboratories. Seed lots B197 and B189 were chosen because of their differences in infection. Seeds were shaken vigorously for 5 minutes in sterile saline with Tween 20, Benlate and Daconil. Dilutions were plated onto NSCA or NSCAA. Table 1 shows means of log cfu of *X. c.* pv. *campestris* obtained by three laboratories. No significant differences were found between NSCA and NSCAA with respect to log cfu of *X. c.* pv. *campestris* (P=0.887). Therefore the values for these media were averaged. ANOVA showed no differences between the treatments with or without a centrifugation step (P=0.464). Therefore, the centrifugation step was omitted in subsequent experiments.

Effect of addition of Tween 20, Benlate and Daconil

To study the effect of Tween 20, Benlate and Daconil, extraction of *X. c.* pv. *campestris* took place by shaking seeds for 5 minutes or 2.5 hours at room temperature, soaking seeds in the refrigerator for 1.5 hours or extracting seeds under vacuum for 5 minutes.

Table 1. The effect of centrifugation on colony counts for *Xanthomonas campestris* pv. *campestris*.

Treatment	Seed lot	
	B189	B197
with centrifugation	0.42[1]	1.56
without centrifugation	0.46	1.76

[1]means of log colony-forming units of *X. c.* pv. *campestris* per ml; standard error of differences of means = 0.24 (number of replications = 60).

Plating was done onto NSCA and NSCAA. Table 2 shows means of log cfu of *X. c.* pv. *campestris* obtained by five laboratories. Since no differences were found between the media NSCA and NSCAA (P=0.408), values of both media were averaged for calculating the means mentioned in table 2.

In general, the treatment without Tween 20, Benlate and Daconil gave significantly higher means for the log cfu of *X. c.* pv. *campestris* per ml than the treatment with Tween 20, Benlate and Daconil. In these experiments 2.5 hours shaking resulted in the highest means of log cfu of *X. c.* pv. *campestris* for seed lot B197. For seed lot B189 there were no or slight differences between extraction methods. The vacuum extraction method gave the lowest means for both seed lots. The addition of Tween 20, Benlate and Daconil to sterile saline was omitted in subsequent experiments. The vacuum extraction method was not further investigated.

Washing time

Three extraction methods, viz. 5 minutes and 2.5 hours shaking of seeds at room temperature and 1.5 hours soaking of seeds were investigated by three laboratories using six seed lots. Plating was done on NSCA and NSCAA. Table 3 shows the effect of three

Table 2. The effect of addition of Tween 20, Benlate and Daconil on colony counts for *Xanthomonas campestris* pv. *campestris* using several extraction methods.

Treatment	Seed lot	Extraction method			
		5 minutes shaking	2.5 hours shaking	1.5 hours soaking	5 minutes vacuum extraction
saline with Tween 20, Benlate and Daconil	B189	0.51[1]	0.31	0.32	0.07
	B197	1.65	2.32	1.38	1.08
saline without Tween 20, Benlate and Daconil	B189	0.44	0.54	0.46	0.28
	B197	1.85	2.88	2.01	1.49

[1]means of log colony-forming units of *X. c.* pv. *campestris* per ml. Standard error of differences of means (s.e.d.) are: 0.20 for comparison of 5 minutes and 2.5 hours shaking, 0.19 for comparison of 5 minutes shaking, 1.5 hours soaking and 5 minutes vacuum, 0.19 for comparison of 2.5 hours shaking, 1.5 hours soaking and 5 minutes vacuum (number of replications = 100).

extraction methods on the log cfu of *X. c.* pv. *campestris* per ml and the log of the total cfu per ml. No significant differences were present between the media NSCA and NSCAA with respect to the log cfu of *X. c.* pv. *campestris* per ml (P=0.786). Therefore both media were included in calculations of means. No interaction was present in any of the comparisons between the extraction methods and seed lot at x20 dilution. ANOVA showed that 2.5 hours shaking gave significantly higher means for the log cfu of *X. c.* pv. *campestris* per ml than 5 minutes shaking (P=0.007). Also 1.5 hours soaking gave significantly higher means than 5 minutes shaking (P=0.003). No significant differences were found between 1.5 hours soaking and 2.5 hours shaking (P=0.754). With respect to

Table 3. The effect of different extraction methods on colony counts for *Xanthomonas campestris* pv. *campestris*.

Treatment	Seed lot					
	B188	B196	B209	B213	B214	B215
5 minutes shaking	0.20[1]	0.51	1.18	0.90	0.26	0.09
	(3.19)	(3.57)	(3.79)	(5.39)	(3.73)	(4.10)
2.5 hours shaking	0.26	0.71	1.70	2.65	0.64	0.05
	(3.42)	(4.03)	(4.29)	(5.67)	(4.35)	(4.65)
1.5 hours soaking	0.30	0.52	1.80	0.58	0.63	0.05
	(3.42)	(3.74)	(4.00)	(5.37)	(3.81)	(4.10)

[1] means of log colony-forming units (cfu) of *X. c.* pv. *campestris* per ml and, between brackets, log cfu of the total number of colonies on NSCAA per ml. Standard error of differences of means (s.e.d.) for log cfu of *X. c.* pv. *campestris* per ml are: 0.21 for comparison of 5 minutes and 2.5 hours shaking, 0.20 for comparison of 5 minutes shaking and 1.5 hours soaking, 0.22 for comparison of 2.5 hours shaking and 1.5 hours soaking (number of replications = 60).

the log of the total cfu per ml, 2.5 hours shaking gave generally higher means than the other two extraction methods.

Table 4 shows the relative number of pathogen colonies as percentage of the total number of colonies (including zero readings). The ratio of pathogen to the total number of colonies did not necessarily decrease or increase with longer extraction times. This greatly depended on the seed lot. For example, for B214 and B209 longer extraction times resulted in higher ratios than a short extraction time. On the other hand, a short extraction time was relatively favourable for B215 and B188. NSCAA gave generally higher ratios than NSCA.

Not only more colonies were found after 1.5 hours soaking and 2.5 hours shaking compared to 5 minutes shaking, but also significant differences between the three extraction methods in the total number of positive (=infected) subsamples per seed lot were found for some seed lots (table 5). Two and a half hours shaking gave for some seed lots, e.g. B213 and B209, significantly more positive subsamples than 5 minutes shaking. The same was valid for 1.5 hours soaking compared to 5 minutes shaking, e.g. for seed lots B209 and B213. The significant effects were no general effects and should therefore be restricted to individual seed lots. No differences were found between media with respect to the number of positive subsamples per seed lot (data not shown). Also no differences were found between either of the extraction methods with respect to the number of positive seed lots (data not shown).

Media

In none of the experiments, described above, have we found significant differences between NSCA and NSCAA with respect to log cfu of *X. c.* pv. *campestris*, number of positive subsamples per seed lot or total number of positive seed lots. However, NSCAA generally showed lower numbers of saprophytic bacteria (starch hydrolysing as well as non-starch hydrolysing bacteria). The FS medium was compared to NSCA and NSCAA in one experiment using 5 minutes shaking of seeds. When 570 subsamples of 114 seed lots were plated on NSCA, NSCAA or FS, fourteen seed lots were found positive with FS and NSCA, twenty seed lots were found positive with NSCAA. Differences between the media with respect to the number of positive seed lots or subsamples were not significant ($0.3 < P < 0.5$, tested by χ^2-test).

Size of (sub)sample

To check whether differences occur in testing five subsamples of 2000 seeds compared to testing one sample of 10 000 seeds, each seed washing was plated in duplicate on NSCA and NSCAA using 5 minutes shaking. In table 6 log cfu of *X. c.* pv. *campestris* per ml are given. The comparison of different sizes of (sub)samples was made by individual laboratories. No significant differences were found between NSCA and NSCAA.

Table 4. Ratio of *Xanthomonas campestris* pv. *campestris* colonies to total number of colonies (percentages).

Treatment	Medium	Seed lot[1]					
		B188	B196	B209	B213	B214	B215
5 minutes	NSCA	0.04±0.32 (70)	2.59±11.21 (77)	4.13±9.73 (70)	1.60±8.75 (23)	0.25±1.67 (71)	0.12±0.84 (56)
shaking	NSCAA	0.19±1.14 (74)	10.82±25.17 (79)	11.89±22.07 (75)	0.07±0.45 (30)	3.93±18.17 (79)	0.11±0.63 (67)
2.5 hours	NSCA	0.01±0.00 (63)	1.19±3.29 (59)	9.21±13.79 (48)	1.85±3.97 (14)	4.46±14.81 (55)	<0.01±0.00 (44)
shaking	NSCAA	0.05±0.45 (71)	7.85±15.82 (75)	14.36±21.59 (61)	1.32±3.41 (17)	8.35±24.01 (63)	0.03±0.00 (54)
1.5 hours	NSCA	<0.01±0.00 (56)	0.12±0.55 (57)	11.06±22.81 (54)	0.46±1.30 (22)	3.79±15.69 (53)	0.11±0.45 (50)
soaking	NSCAA	0.04±0.32 (58)	0.40±1.10 (63)	14.12±19.26 (62)	0.73±2.30 (23)	7.84±22.06 (61)	0.04±0.32 (58)

[1] Values are mean ratios ± standard deviation (in percentages) of plates with less than 250 colonies at three serial dilutions; between brackets the percentages of plates with less than 250 colonies. For 5 minutes and 2.5 hours shaking at room temperature 150 plates were used. For 1.5 hours soaking in the refrigerator 90 plates were used.

Therefore, values for these media were averaged. No significant differences were found between testing 5 x 2000 seeds or 1 x 10 000 in log cfu of *X. c.* pv. *campestris* per ml or in number of positive seed lots (resp. tested by Wilcoxon matched-pairs signed-ranks test on means given in table 6 and by a χ^2 test on the number of positive seed lots, derived from table 6).

Discussion

In this study several aspects of the plating assay decribed by Schaad (1982) were evaluated. Preliminary experiments with two seed lots showed no positive effect of a centrifugation step prior to plating (table 1). Theoretically, centrifugation of seed washings may be useful for detecting very low levels of infection. However, centrifugation is only useful when the level of contamination with *X. c.* pv. *campestris* is lower than 20 cfu/ml (which corresponds to one cfu of *X. c.* pv. *campestris* in the undiluted seed washing when 50 μl is plated onto a 9 cm petri dish) and when the contamination with saprophytes is less than 5000 cfu/ml (which corresponds to 250 cfu in the undiluted seed washing when 50 μl is plated onto a 9 cm petri dish). Saprophyte to pathogen ratios should not exceed 250 (in our study). We have not found seed lots which meet these requirements, yet. However, we realize that seed lots can differ between seasons and between growing areas in numbers of saprophytic bacteria and in numbers of *X. c.* pv. *campestris* colonies. For routine application we advise omitting a concentration step since this is a laborious part of a routine assay.

The negative effect of addition of Tween 20, Benlate and Daconil (table 2) is probably only due to the bacteriostatic action of Daconil on *X. c.* pv. *campestris*. The addition of Benlate is in general not necessary because the cycloheximide in the medium suppresses the fungi adequately. Addition of Tween 20 may be useful especially for short washings (ISTA, 1982), but is probably not necessary for longer washing times. For routine indexing of seed lots we advise omitting the addition of Tween 20, Benlate and Daconil to saline.

Some of the extraction methods used in this study were earlier used in ISTA

Table 5. The effect of different extraction times on the number of positive subsamples per seed lot.

Treatment	Seed lot					
	B188	B196	B209	B213	B214	B215
5 minutes shaking	0.50[1]	1.83	2.67	1.00	1.17	0.83
2.5 hours shaking	0.33	2.00	3.50	2.17	1.33	0.50
1.5 hours soaking	0.50	2.41	4.50	1.50	1.00	0.67

[1]Mean number of positive (infected) subsamples per seed lot when testing seed lots in five subsamples of 2000 seeds. Standard error of differences of means (s.e.d.) are: 0.35 for comparison of 5 minutes and 2.5 hours shaking, 0.42 for comparison of 5 minutes shaking and 1.5 hours soaking, 0.42 for comparison of 2.5 hours shaking and 1.5 hours soaking (number of replications = 6).

Table 6. Comparison of testing five subsamples of 2000 seeds to testing one sample of 10 000 seeds per seed lot.

Seed lot	Size of sample	Colony counts[1] for *Xanthomonas campestris* pv. *campestris*
B188	5 x 2000 seeds	0.00 ± 0.00 (20)
	1 x 10 000 seeds	0.00 ± 0.00 (4)
B196	5 x 2000 seeds	0.27 ± 0.85 (20)
	1 x 10 000 seeds	3.04 ± 0.09 (4)
B209	5 x 2000 seeds	0.39 ± 0.95 (20)
	1 x 10 000 seeds	0.00 ± 0.00 (4)
B214	5 x 2000 seeds	0.31 ± 0.97 (20)
	1 x 10 000 seeds	1.30 ± 1.50 (4)
B215	5 x 2000 seeds	0.13 ± 0.58 (20)
	1 x 10 000 seeds	0.00 ± 0.00 (4)
X39	5 x 2000 seeds	0.00 ± 0.00 (20)
	1 x 10 000 seeds	0.00 ± 0.00 (4)
X43	5 x 2000 seeds	0.13 ± 0.58 (20)
	1 x 10 000 seeds	0.00 ± 0.00 (4)
X103	5 x 2000 seeds	2.69 ± 1.86 (20)
	1 x 10 000 seeds	3.89 ± 0.06 (4)
X109	5 x 2000 seeds	1.47 ± 1.54 (20)
	1 x 10 000 seeds	3.47 ± 0.20 (4)
X115	5 x 2000 seeds	2.12 ± 1.62 (20)
	1 x 10 000 seeds	0.00 ± 0.00 (4)
X120	5 x 2000 seeds	0.00 ± 0.00 (20)
	1 x 10 000 seeds	0.00 ± 0.00 (4)
X149	5 x 2000 seeds	0.00 ± 0.00 (20)
	1 x 10 000 seeds	0.00 ± 0.00 (4)

[1] Means of log colony-forming units of *X. c.* pv. *campestris* per ml. Between brackets number of replications (each (sub)sample was plated in duplicate onto NSCA and NSCAA).

comparative tests. Two and a half hours shaking was first investigated at the ISTA Bacteriology workshop in 1985 in Copenhagen (ISTA, unpublished). One and a half hours soaking of seeds was suggested by C. van Henten (personal communication) and used in an ISTA comparative test (ISTA, 1988). The results of the ISTA comparative test showed that longer washing times could increase the number of cfu/ml of *X. c.* pv. *campestris*. This depended, however, on the laboratory and seed lot. During this study other reports also suggested that longer washing times may increase the mean cfu/ml (Chang et al., 1989a; Chang et al., 1989b). Short washings (5 minutes) are generally considered to work satisfactory (Schaad, 1989). Also in our study we did not find general differences between extraction methods with respect to the number of positive seed lots. However, for some seed lots the number of positive subsamples per seed lot may increase when using longer washings. Therefore, it may be advisable to apply longer washings (e.g. 2.5 hours shaking) for some seed lots. This may increase the success of isolation. It may also avoid the necessity of a centrifugation step. Moreover, when higher numbers of *X. c.* pv. *campestris* colonies are recovered after longer washings it is easier to find a seed lot which is infected with *X. c.* pv. *campestris*. Based on these findings seed lots are presently tested in the Netherlands by applying 5 minutes and 2.5 hours shaking of seed lots.

The media used in this study, NSCA and NSCAA, were chosen because of their simplicity in preparation. Further, colonies appear more rapidly on these media than on media like BSCAA, FS and CS20ABN. The performance of the media depends strongly on the seed lot to be tested (ISTA, 1988; Schaad, 1989). The saprophytic population may vary in level and composition per seed lot. Therefore it is important to use several media for routine-indexing of seed lots for presence of *X. c.* pv. *campestris*. We prefer to use NSCA and NSCAA because they are well suited for routine application. When using longer washing times, another medium like FS may be included (ISTA, 1988; Schaad, 1989).

However, since the performance of the media strongly depends on the seed lot to be tested, it is probably more advisable to combine a plating assay with an assay based on another principle, e.g. immunofluorescence microscopy (IF). The latter assay may detect

low infection levels independently of high saprophyte numbers. We are now in the process of developing a suitable IF procedure, using monoclonal antibodies, for detecting *X. c.* pv. *campestris* in crucifer seeds.

Research is needed to explain interactions of seed lots with plating media. An analysis of bacterial flora in seed lots is therefore probably neccessary. Moreover, it may be important to determine the exact part of the seed which is contaminated with saprophytes and *X. c.* pv. *campestris*. Also, information is needed on the behaviour of *X. c.* pv. *campestris* in plating assays in relation to the growth conditions of the *X. c.* pv. *campestris* cells and epiphytic survival in or on the seed. This research may help to control and predict interactions of seed lots with plating media.

In our study the sensitivity of the test was not higher when testing 5 x 2000 seeds instead of testing 1 x 10 000 seeds. Also no improvement in sensitivity was obtained when seed lots were tested in subsamples of 3 x 3000 seeds, 2 x 5000 seeds and 10 x 1000 seeds using 5 minutes washing times (results not shown). Therefore, (sub)samples of 10 000 seeds can reliably be used to detect *X. c.* pv. *campestris* in cabbage seeds. Since a tolerance of 1 to 3 infected seeds per 10 000 seeds is established for direct seeding of cabbage (Schaad et al., 1980), more than 10 000 seeds should be tested to establish freedom of *X. c.* pv. *campestris* in 10 000 seeds with a high probability. We have chosen to routinely test 30 000 seeds per seed lot in three subsamples of 10 000 seeds in the Netherlands. When three subsamples of a seed lot are found to be negative, the maximum infection level of the seed lot is 0.01% (upper limit) at 95% probability (calculated by a computer program to estimate the percentages of infected seeds when testing seed lots in several subsamples, as reported by Van Vuurde and Maat (1983)). More probably the infection level is usually much lower than 0.01% when all subsamples are found negative.

A flow diagram of the plating assay, presently used in the Netherlands for *X. c.* pv. *campestris* is given in figure 1.

Figure 1. Flow diagram for detecting *Xanthomonas campestris* pv. *campestris* by a plating assay in seeds of crucifers.

Weight three times 10 000 seeds

↓

Add 100 ml of 0.85% NaCl to each subsample of 10 000 seeds

↓

Shake for 5 minutes and 2.5 hours at 21 °C

↓

Take sample of 1 ml and dilute serially to 1:100 and plate 50 µl of 0, 1:10 and 1:100 dilutions onto duplicate plates of NSCA/FS and NSCAA. Incubate plates at 25°C and observe suspected colonies after three days (NSCA and NSCAA) or five days (FS)

↓

Confirm suspected colonies of *X. c.* pv. *campestris* onto YDC and in a pathogenicity test (and possibly immunofluorescence microscopy)

References

Chang, C.J., Donaldson, R., Crowley, M. and Pinnow, D. (1989a). Isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds using a highly selective medium. Abstracts of papers and posters, pp. 147. 7th International Conference on Plant Pathogenic Bacteria, 1989, Budapest, Hungary.

Chang, C.J., Donaldson, R., Crowley, M. and Pinnow, D. (1989b). Comparison of the isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds in semiselective media (abstract). Phytopathology 79: 1140.

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.

ISTA (1982). Report on the 1st International Workshop on Seed Bacteriology, 1982, Angers, pp. 45. International Seed Testing Association, Zürich, Switzerland.

ISTA (1988). Report from 19th International Seminar on Seed Pathology, 1987, Wageningen, pp. 13-14. International Seed Testing Association, Zürich, Switzerland.

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. (1982). *Xanthomonas campestris* pv. *campestris*. Working sheet no. 50. ISTA handbook on seed health testing. International Seed Testing Association, Zürich, Switzerland.

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

Schaad, N.W. and Kendrick, R. (1975). A qualitative method for detecting *Xanthomonas campestris* in crucifer seeds. Phytopathology 65: 1034-1036.

Schaad, N.W. and White, W.C. (1974). A selective medium for soil isolation and enumeration of *Xanthomonas campestris*. Phytopathology 64: 876-880.

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: 91-92.

Siegel, S. (1956). Nonparametric statistics for the behavioral sciences. McGraw-Hill Book Company, Inc., Tokyo, pp. 312.

Van Vuurde, J.W.L. and Maat, D.Z. (1983). Routine application of ELISA for the detection of lettuce mosaic virus in lettuce seeds. Seed Science and Technology 11: 505-513.

Van Vuurde, J.W.L., Van den Bovenkamp, G.W. and Birnbaum, Y.E. (1983). Immunofluorescence microscopy and enzyme-linked immunosorbent assay as potential routine test for the detection of *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* in bean seed. Seed Science and Technology 11: 547-558.

Chapter 4. Specificity of polyclonal and monoclonal antibodies for the identification of *Xanthomonas campestris* pv. *campestris*

Specificity of polyclonal and monoclonal antibodies for the identification of *Xanthomonas campestris* pv. *campestris*

Abstract

Polyclonal and monoclonal antibodies (PCAs and MCAs), produced to whole cells and flagellar extracts of *Xanthomonas campestris* pv. *campestris* (Xcc), respectively, were tested for specificity. In immunofluorescence microscopy (IF) the three PCAs tested, reacted at low dilutions with all Xcc strains, some other xanthomonads and non-xanthomonads. At higher dilutions most cross-reactivity with non-xanthomonad strains disappeared. However, the cross-reactivity with strains of *X. c.* pv. *vesicatoria* (Xcv), *X. c.* pv. *amoraciae* (Xca) and *X. c.* pv. *phaseoli* var. fuscans (Xcpf) remained.

Six MCA-producing cell clones viz. 20H6, 2F4, 18G12, 10C5, 17C12 and 16B5 were selected for specificity tests with an enzyme immunoassay (EIA), IF and a dot-blot immunoassay (DBI). None of the MCAs reacted with all Xcc strains in IF and EIA. In DBI, only MCAs 17C12 and 16B5 reacted with all Xcc strains. All six MCAs tested, cross-reacted in one of either tests with other pathovars of *X. campestris*, such as Xcv or Xca. The MCAs were also tested in immunoblotting experiments using total bacterial extracts, cell envelope and flagellar extracts. MCAs 20H6, 2F4, 18G12 and 10C5 reacted with the lipopolysaccharide (LPS) of Xcc. MCAs 16B5 and 17C12 reacted with a 39 kilodalton and a 29 kilodalton protein, respectively.

It is concluded that the PCAs and MCAs discussed in this study may be used for routine identification and differentiation of (a group of) Xcc strains. The significance of the cross-reactions with other pathovars of *X. campestris* needs to be determined by testing seed lots.

Additional keywords: black rot, serology, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), proteinase K.

Introduction

Xanthomonas campestris pv. *campestris*, the causal agent of black rot in crucifers, is generally considered the most important disease of crucifers in the world (Williams, 1980). The use of healthy seeds is an important way to prevent the black rot disease. Methods to detect this pathogen in seeds are therefore needed. Serological techniques are potentially very sensitive and specific for detection and identification of seed-borne bacteria. Therefore high-quality antibodies are needed. Both polyclonal and monoclonal antibodies (PCAs and MCAs, respectively) are used in seed bacteriology although the application of monoclonal antibodies is still limited (Franken and Van Vuurde, 1990). MCAs for *X. c.* pv. *campestris* (Xcc) were developed by Alvarez et al. (1985). These antibodies were tested for application in a radio-immunoassay and an enzyme immunoassay (EIA), and for identification and grouping of strains (Alvarez et al., 1985; Alvarez and Lou, 1985; Yuen et al., 1987). Haaheim et al. (1989) produced monoclonal antibodies that reacted with the exopolysaccharide xanthan from *X. campestris*.
The aim of this study was to evaluate the specificity of PCAs and MCAs, produced in our laboratory against Xcc.

Material and methods

Organisms and growth media
The strains employed in this study are listed in table 1. All bacteria were cultured on a solid growth factor (GF) medium, unless otherwise stated, containing per l: 0.4 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.5 g $NH_4H_2PO_4$, 0.01 g $FeCl_3$, 1.0 g glucose, 3.0 g yeast extract and 15.0 g Bacto-agar. All strains were grown at 27-28 °C.

Enzyme immunoassay (EIA)
Polystyrene microplates were coated for 30 min at 21 °C with poly-L-lysine, 0.1 mg ml^{-1}, in carbonate buffer, pH 9.6 (Clark and Adams, 1977). Plates were washed 3 times with

Table 1. Bacterial strains employed for quality-testing of monoclonal and polyclonal antibodies produced against *Xanthomonas campestris* pv. *campestris*.

Bacteria	Strain designation	Received from[1]
Clavibacter michiganensis subsp. *michiganensis*	Cmm 542	4
Clavibacter michiganensis subsp. *insidiosus*	Cmi 533	4
Curtobacterium flaccumfaciens subsp. *flaccumfaciens*	Cff 547	4
Erwinia herbicola	Eher 732, Eher 1062, Eher 1063,	4
	Eher 83	2
Pseudomonas aeruginosa	Paer 60	4
Pseudomonas marginalis	Pmar 86/1118	2
Pseudomonas viridiflava	Pvir 540	4
Pseudomonas cichorii	Pcich 478	2
Pseudomonas syringae pv. *lachrymans*	Psl 520	4
Pseudomonas syringae pv. *maculicola*	Psm 154, Psm 155	4
Pseudomonas syringae pv. *phaseolicola*	Psp 570, Psp 111	4
Pseudomonas syringae pv. *pisi*	Pspi 518, Pspi 519	4
Pseudomonas syringae pv. *syringae*	Pss 1147, Pss 126	4,5
Xanthomonas campestris pv. *amoraciae*	Xca 373, Xca 374	4
Xanthomonas campestris pv. *carotae*	Xcar	11
Xanthomonas campestris pv. *campestris*	Xcc 102, Xcc 297, Xcc 298, Xcc 363, Xcc 364, Xcc 365, Xcc 366, Xcc 367, Xcc 368, Xcc 369, Xcc 370, Xcc 371, Xcc 372[2], Xcc 557, Xcc 558, Xcc 559, Xcc 634, Xcc 671,	4
	Xcc 8-6, Xcc 2-24, Xcc 2-48, Xcc 4-4, Xcc 9-48, Xcc 9-4, Xcc 9-30N, Xcc 9-30Z,	1
	Xcc 3-31-8, Xcc 2-9-2, Xcc 8-19-1,	9
	Xcc BT-7, Xcc BT-8,	10
	Xcc 46-6, Xcc 117-26, Xcc 824-1,	8
	Xcc 46 Cop.	7
	Xcc SG 11, Xcc SG 57	6
Xanthomonas campestris pv. *phaseoli*	Xcp 375, Xcp 510	4
Xanthomonas campestris pv. *phaseoli* var. fuscans	Xcpf 382, Xcpf 482	4
Xanthomonas campestris pv. *vesicatoria*	Xcv 512, Xcv 523	4
Xanthomonas maltophilia	Xm 1974, Xm 1975	3
unidentified	saprophyte 457	1

[1] 1=Authors; 2=Plant Protection Service, the Netherlands; 3=National Culture Collection for Plant Pathogenic Bacteria, England; 4=Research Institute for Plant Protection, the Netherlands; 5= B. Rat, France; 6= C. van Henten, Netherlands; 7= F. Vinther, Denmark; 8= P.H. Williams, Wisconsin, USA; 9= D.L. Pinnow, Georgia, USA; 10= N. Thaveechai, Thailand; 11= T.L. Kuan, Washington, USA.

[2] Later designated as *X. c.* pv. *aberrans*.

phosphate-buffered saline (PBS), containing 0.1 % Tween 20 (PBST). Antigens were untreated or boiled cells at a concentration of 10^7 cells ml^{-1} and bacterial extracts (10.0, 1.0 and 0.1 μg protein per ml in PBS). Plates were incubated with antigen overnight at 4 °C and washed 3 times with PBST. Blocking took place by incubation with PBST, containing 0.5% bovine serum albumin, for 30 min at 37 °C. Antibodies from hybridoma supernatants (undiluted in culture medium) were added and incubated for 3 h at 4 °C; PCAs were not tested. Plates were washed 3 times with PBST. Goat-anti-mouse alkaline phosphatase-conjugate (GAM-AP) was added in a 1:1000 dilution in PBST, containing 0.1 % albumin, and incubated for 2 h at 37 °C. Plates were then washed 3 times with PBST and subsequently incubated with enzyme substrate (0.75 mg ml^{-1} p-nitrophenyl phosphate in 10% diethanolamine buffer (v/v), pH 9.8) for 0.5-1 h at 37 °C. Absorbance values were read at 405 nm.

Dot-blot immunoassay (DBI) and immunoblotting

The procedure for DBI was as described for EIA, with some modifications. One μl of antigen suspension was spotted onto nitrocellulose membranes (Schleicher & Schüll, 0.45 μm). The membranes were dried, blocked with PBS containing 0.5% horse serum, washed three times, probed with antibodies from hybridoma supernatants (PCAs were not tested) for 2 h at 21 °C, washed three times and incubated with GAM-AP for 1 h at 21 °C. The reaction was visualized with 5 mg nitroblue tetrazolium in 5 ml deionized water to which 0.75 ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution (4 mg ml^{-1} in ethanol/acetone solution, 2:1) was added, and which was totally dissolved in 0.1M ethanolamine-4mM $MgCl_2$ buffer, pH 9.6. Immunoblotting of SDS-PAGE gels was carried out as described by Boonekamp et al. (1990); blots were developed with antibodies, GAM-conjugate and BCIP as described above for DBI.

Immunofluorescence microscopy (IF)

Indirect IF was carried out as described by Van Vuurde et al. (1983). Antibodies from hybridoma culture supernatants were used undiluted. Secondary goat-anti rabbit antibodies, to which fluorescein-isothiocyanate was conjugated (Sigma), were used at a

1:100 dilution.

Preparation of bacterial extracts for the production of MCAs and quality testing of MCAs and PCAs

Flagellar extracts were prepared from cells in the early stationary phase ($\pm$ 10^8-10^9 cells ml^{-1}), using the shearing and differential centrifugation methods essentially as described by Martin and Savage (1985). In this procedure flagella were separated from cell debris by two differential centrifugation steps at 5000 g and 15 000 g. The two residual fractions containing the cell debris were used to prepare cell envelope extracts. These fractions were resuspended in a 50mM Tris - 2mM EDTA buffer, pH 8.5 and pooled. The pooled suspension was placed on ice and repeatedly sonicated in short bursts on ice with a Killings apparatus. After sonic disruption, large fragments were removed by centrifugation at 2000 g for 20 min at 4 °C. The supernatant fluid was centrifuged at 230 000 g (at r_{max}) for 2 h at 4 °C and the pellet was resuspended in 5 ml PBS. This suspension was called the cell envelope extract.

Total bacterial extracts were prepared from cells in the early stationary phase, as described by Kiredjian et al. (1986). Cells were suspended in sample treatment buffer with sodium-dodecyl sulphate (see below) and heated for 10 min at 100 °C. The suspension was centrifuged for 5 min at 13 000 g. The supernatant fluid was called the total bacterial extract.

Proteinase K treatment of cell envelope, flagellar and total bacterial extracts was done as described by De Weger et al. (1987).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE using reducing conditions was done as described by Laemmli (1970). A 4% stacking gel and 12.5% separating gel were used. Samples containing 5 - 10 μg protein were diluted with an equal volume of a 2x sample buffer. Gels were run at 200 V, and used for electroblotting to nitrocellulose membranes, staining with 0.025% (w/v) Coomassie Brilliant Blue R or staining with silver for the lipopolysaccharide (Tsai and Frasch, 1982).

Production of antibodies

PCAs were produced by H. Vruggink, IPO-DLO (Research Institute for Plant Protection, Wageningen, the Netherlands), against whole living cells. Cells were washed twice in PBS and centrifuged for 10 min at 6000 g prior to immunization. PCA 94 was produced against Xcc strain 367, PCA 111 against Xcc strain 102, PCA 113 against Xcc strain 365.

For production of MCAs, BALB/c mice were immunized three times, intraperitoneally, with flagellar extracts of strain Xcc 367 (20 μg). The first immunization was in Freund's complete adjuvant and the second, ten to fourteen days later, in Freund's incomplete adjuvant. Fourteen days after the second immunization the titre was determined by EIA. If necessary a third immunization of antigen in PBS was given.

Fusion and culturing of hybrid cell lines was done as described by Boonekamp et al. (1990).

Selection of hybridoma lines

Hybridoma lines were first screened in EIA with cell envelope extracts and flagellar extracts of Xcc strains 367, 671 and 102 and afterwards with whole cells of a selected number of bacterial strains (Xcc 102, Xcc 367, Xcc 671, Xcv 523, Xcpf 382, Xcp 375, Psm 154, Psp 570, Cmm 542 and saprophyte 457, Table 1). A third screening was done with selected MCAs in EIA, DBI and IF using a wider range of bacterial genera and other Xcc isolates. For this screening, cells were heated for 15 min at 100 °C, prior to testing by EIA and DBI, to diminish background reactions of some bacteria. Isotyping of the MCAs was carried out using the Biorad mouse sub-isotyping kit in EIA and agar double diffusion.

Results

Specificity of PCAs in IF

The specificity of PCAs was studied in IF using dilutions of 1:100, 1:300, 1:900, 1:2700 and 1:8100. Strains of Xcc, other pathovars of *X. campestris* and several

Table 2. Reactions of polyclonal antibodies prepared against *Xanthomonas campestris* pv. *campestris* with strains of various bacterial genera in immunofluorescence microscopy.

Designation of polyclonal antibodies	Bacteria[1]	Number of strains positive / Number of strains tested at three dilutions		
		1:100	1:900	$\geq$ 1:8100
94	Xcc	37/37	27/37	7/37
	Xc pvs.	5/6	3/6	0/6
	other	0/22	0/22	0/22
111	Xcc	37/37	20/37	0/37
	Xc pvs.	6/6	0/6	0/6
	other	3/22	0/22	0/22
113	Xcc	37/37	24/37	12/37
	Xc pvs.	5/6	2/6	0/6
	other	4/22	1/22	0/22

[1] Xcc = *Xanthomonas campestris* pv. *campestris*; Xc pvs. = other pathovars of *X. campestris*: *X. c.* pv. *phaseoli* (two strains), *X. c.* pv. *phaseoli* var. fuscans (one strain) and *X. c.* pv. *vesicatoria* (two strains) and *X. c.* pv. *amoraciae* (one strain). Other are non-xanthomonads: *Pseudomonas syringae* pv. *phaseolicola* (2 strains), *P. s.* pv. *syringae* (2 strains), *P. s.* pv. *maculicola* (2 strains), *P. s.* pv. *pisi* (2 strains), *P. s.* pv. *lachrymans* (1 strain), *P. aeruginosa* (1 strain), *P. viridiflava* (1 strain), *P. cichorii* (1 strain), *P. marginalis* (1 strain), *Erwinia herbicola* (3 strains), *Clavibacter michiganensis* subsp. *michiganensis* (1 strain), *Cl. m.* subsp. *insidiosus* (1 strain), *Curtobacterium flaccumfaciens* subsp. *flaccumfaciens* (1 strain), *X. maltophilia* (2 strains) and saprophyte 457.

non-xanthomonads (whole cells) were tested. Table 2 shows that at dilution 1:100, PCAs 94, 111 and 113 cross-reacted with other pathovars of *X. campestris* (pv. *vesicatoria* (Xcv), pv. *amoraciae* (Xca) and pv. *phaseoli* var. fuscans (Xcpf)). PCAs 111 and 113 also cross-reacted with some non-xanthomonads at this dilution. The cross-reacting non-xanthomonad strains were: Psm 154 and 155, Eher 1062 and 1063, Pcich 478 and

saprophyte 457, isolated from cabbage seed. At dilutions 1:900 most of the cross-reactions disappeared, but saprophyte 457 still cross-reacted with PCA 113. However, at dilutions of 1:900 and 1:8100 none of the PCAs reacted anymore with all Xcc strains tested. Some strains of other pathovars of *X. campestris* (Xcv, Xca) reacted as well as strains of Xcc in IF.

The homologous strains Xcc 102, 365 and 367 each reacted strongest with their homologous antiserum. Xcc 102 reacted up to a dilution of 1:2700 with PCA 111 and up to 1:300 with PCAs 113 and 94. Xcc 365 reacted up to a dilution of 1:8100 with PCA 113, whereas it reacted up to 1:100 and 1:2700 with PCAs 111 and 94, respectively. Xcc 367 reacted up to a dilution of 1:8100 with PCA 94, whereas it reacted up to 1:300 and 1:900 with PCAs 111 and 113, respectively. Only a few strains reacted equally well with all three PCAs. For example, Xcc 671 reacted well up to a 1:900 dilution with all three PCAs.

Reaction of PCAs in immunoblotting

The reactions of PCAs 94, 111 and 113 with antigens in cell envelope extracts of several Xcc strains and saprophyte 457 was studied by SDS-PAGE and immunoblotting (fig. 1). The 44, 39, 25, 23, 18 and 17 kilodalton (kDa) bands of several Xcc strains reacted with the PCAs tested. Each PCA reacted strongest with antigenic components of the homologous strain in the region above 67 kDa. This smear was assumed to be the LPS since proteinase K treatment did not affect the reaction in immunoblotting (see even numbered lanes) and since LPS was stained in gels at the same position (data not shown). The strong reaction with the LPS of the homologous strains indicates that differences between the Xcc strains in the LPS are present. PCAs 94 and 113 also reacted relatively strong with the 23 and 18 kDa bands and the LPS of saprophyte 457 (lanes 7). Although PCA 94 did not cross-react significantly with saprophyte 457 in IF (table 2), it reacted with the LPS of saprophyte 457 in this test, although weaker than with the LPS of the Xcc strains. On the other hand, PCA 111 did not clearly react with the LPS of saprophyte 457 although cross-reactions were found with saprophyte 457 at a 1:100 dilution in IF.

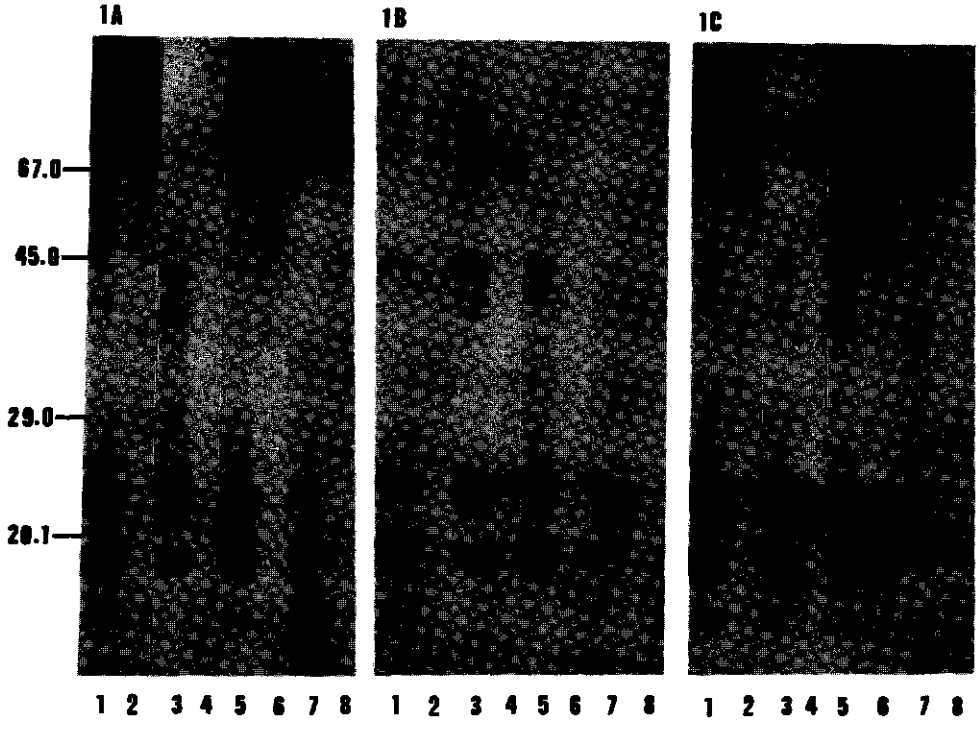

Fig. 1. Immunoblotting with polyclonal antibodies raised against *Xanthomonas campestris* pv. *campestris* to identify bands of cell envelope extracts, separated by SDS-PAGE.
A = immunoblot probed with PCA 94 (homologous strain Xcc 367).
B = immunoblot probed with PCA 111 (homologous strain Xcc 102).
C = immunoblot probed with PCA 113 (homologous strain Xcc 365).
Lanes 1,2: Cell envelope extracts of Xcc 367.
Lanes 3,4: Cell envelope extracts of Xcc 102.
Lanes 5,6: Cell envelope extracts of Xcc 365.
Lanes 7,8: Cell envelope extracts of saprophyte 457.
Uneven lanes: cell envelope extracts not treated with proteinase K.
Even lanes: cell envelope extracts treated with proteinase K.
Relative molecular masses (kilodaltons) of standard proteins are indicated to the left of the figures.

Characterization of flagellar extracts with PCAs

In SDS-PAGE, flagellar extracts seemed primarily to contain a 58 kDa protein and 44 kDa protein (fig. 2). However, on immunoblots with PCAs 94, 111 and 113 bands were observed in the region of 15, 18, 23, 29 (weak reaction for PCA 113 and Xcc 367 and 671 only), 39, 44 and 58 kDa (fig. 3). The reaction with the smear above 67 kDa indicates that LPS was present in the flagellar extracts.

Reaction of monoclonal antibodies in EIA, IF and DBI

Fourteen stable clones from 2 fusions were selected after subculturing and screening with EIA using flagellar and cell envelope extracts as antigens. After a second screening with EIA, using whole cells of 10 strains of several genera, six clones were selected on the basis of positive reactions with (some of the) Xcc strains and negative reactions with saprophyte 457. These clones were further tested in EIA, IF and DBI with a wider range of strains. Results are shown in table 3. In EIA, DBI and IF, none of the six MCAs cross-reacted with non-xanthomonad strains. For MCAs 16B5 and 17C12, considerable

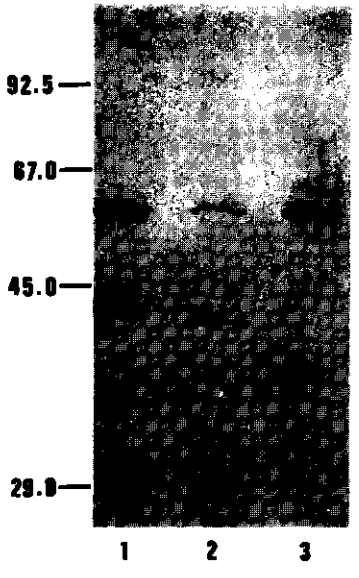

Fig. 2. SDS-polyacrylamide gel electrophoresis of flagellar extracts of several strains of *Xanthomonas campestris* pv. *campestris*. Gels are stained with Coomassie Brilliant Blue.
Lane 1 = flagellar extracts of Xcc 367.
Lane 2 = flagellar extracts of Xcc 102.
Lane 3 = flagellar extracts of Xcc 671.
Relative molecular masses (kilodaltons) of standard proteins are indicated to the left of the figure.

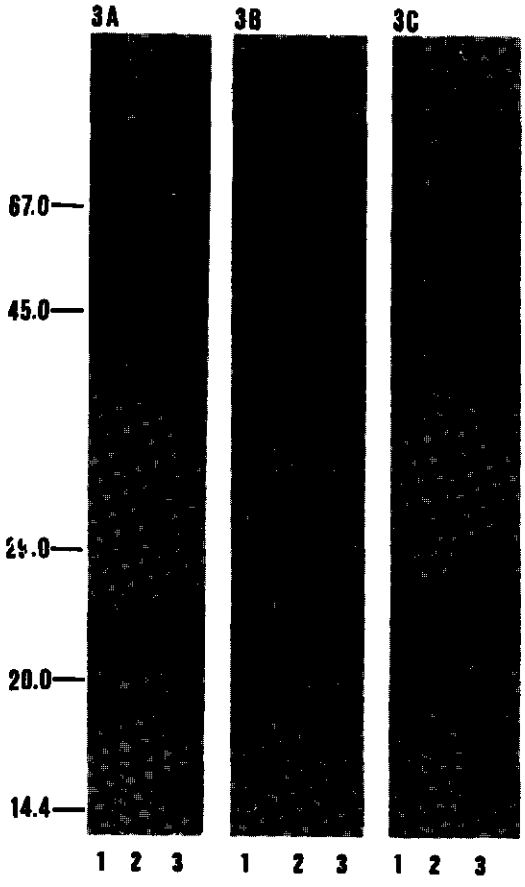

Fig. 3. Immunoblotting with polyclonal antibodies raised against *Xanthomonas campestris* pv. *campestris* to identify bands of flagellar extracts, separated by SDS-PAGE.
A = blot probed with PCA 94.
B = blot probed with PCA 111.
C = blot probed with PCA 113.
Lanes 1 contain flagellar extract of Xcc 367.
Lanes 2 contain flagellar extract of Xcc 102.
Lanes 3 contain flagellar extract of Xcc 671.
Relative molecular masses (kilodaltons) of standard proteins are indicated to the left of the figure.

differences were found between the serological assays used. All tested Xcc strains reacted in DBI whereas only some of the Xcc strains reacted in EIA and IF. All other xanthomonads (other pathovars of *X. campestris*) tested also reacted in DBI with MCAs 16B5 and 17C12, whereas in EIA and IF some strains of other xanthomonads gave negative reactions. For MCAs 20H6, 2F4, 18G12 and 10C5, only some of the Xcc strains reacted in EIA, DBI and IF. Other pathovars of *X. campestris* cross-reacted with MCAs 20H6, 2F4, 18G12 and 10C5 in all three serological tests.

The reactions in IF were studied in more detail. The total number of strains reacting in IF with the MCAs as well as the number of strains of which the whole cell wall was

clearly stained in IF was recorded (table 3). For MCA 16B5, good staining of the whole cell wall was observed for 20 out of 20 Xcc strains reacting in IF. For MCA 2F4, good staining of the whole cell wall was observed for only 9 Xcc strains; for 16 Xcc strains a partial staining of the cell wall was observed in IF. Also for the other MCAs, only a part of the Xcc strains reacting in IF gave good staining of the whole cell wall. The staining quality (fluorescence intensity) in IF was the best for cells stained with MCA 20H6 (not shown). Some strains of other pathovars of *X. campestris*, such as Xcv, reacted also very well with the MCAs. Additional IF tests showed that MCAs 16B5, 17C12, 20H6, 2F4, 18G12 and 10C5 reacted well with the whole cell wall of, at least, one of the Xca strains tested.

Specificity of MCAs in immunoblotting

Total bacterial extracts of Xcc 367, 102 and 671, Xcar, Xcv 523, Xcp 375 and Xcpf 482 and a flagellar extract of Xcc 367, were probed with several MCAs in immunoblotting. Two 'types' of reactions were found.

First, MCAs reacting with the LPS of the homologous Xcc strain 367, viz. MCAs 2F4, 18G12, 10C5 and 20H6 (proteinase K treatment of the extracts did not eliminate the reaction of these MCAs). Only, the blot for MCA 20H6 is shown in fig. 4A and demonstrates a reaction with the LPS of a flagellar and total bacterial extract of Xcc strain 367 (resp. lane 1 and 2, the region above 58.1 kDa). A reaction in the same region was also found in cell envelope extracts (data not shown). MCA 20H6 did not show a reaction in the LPS region of the other strains tested in immunoblotting (lanes 3-8). MCAs 2F4, 18G12 and 10C5, however, also demonstrated a weak reaction with the LPS of Xcv 523 (not shown). MCA 2F4 also reacted with the LPS of Xcc strain 671 (not shown).

Second, MCAs reacting with protein epitopes, viz. MCAs 16B5 and 17C12 (proteinase K treatment of the extracts eliminated the reaction of the MCAs). MCA 16B5 (fig. 4B) reacted with a major c. 39 kDa band of Xcc 367, Xcc 102, Xcc 671, Xcar, Xcv 523, Xcp 375 and Xcpf 482 (lanes 2-8). The reaction with this band also occurred in flagellar extracts (lane 1, fig. 4B) and cell envelope extracts of Xcc strain 367 (not shown). A

Table 3. Reactions of monoclonal antibodies, produced against *Xanthomonas campestris* pv. *campestris*, with strains of various bacterial genera.

Monoclonal antibody	Antibody isotype	Bacteria[1]	Number of strains tested	Number of strains positive			
				EIA[2]	DBI[2]	IF	
						+[3]	±[4]
16B5	IgG2a	Xcc	37	18	37	20	0
		Xc pvs.	6	4	6	3	1
		other	15	0	0	0	0
17C12	IgG2a	Xcc	37	24	37	18	5
		Xc pvs.	6	5	6	3	0
		other	15	0	0	0	0
20H6	IgG1	Xcc	37	27	27	15	6
		Xc pvs.	6	2	1	1	1
		other	15	0	0	0	0
2F4	IgG3	Xcc	37	34	33	9	16
		Xc pvs.	6	2	2	2	0
		other	15	0	0	0	0
18G12	IgG3	Xcc	37	33	32	13	17
		Xc pvs.	6	2	2	2	0
		other	15	0	0	0	0
10C5	IgM	Xcc	37	33	26	13	15
		Xc pvs.	6	2	2	2	0
		other	15	0	0	0	0

[1] Xcc = *Xanthomonas campestris* pv. *campestris*, Xc pvs. = *X. c.* pv. *phaseoli* (2 strains), *X. c.* pv. *phaseoli* var. fuscans (2 strains), *X. c.* pv. *vesicatoria* (2 strains). Other strains are non-xanthomonads: *Pseudomonas syringae* pv. *phaseolicola* (2 strains), *P. s.* pv. *pisi* (2 strains), *P. s.* pv. *syringae* (2 strains), *P. s.* pv. *lachrymans* (1 strain), *P. s.* pv. *maculicola* (2 strains), *P. viridiflava* (1 strain), *P. aeruginosa* (1 strain), *Curtobacterium flaccumfaciens* subsp. *flaccumfaciens* (1 strain), *Clavibacter michiganensis* subsp. *michiganensis* (1 strain), *Cl. m.* subsp. *insidiosus* (1 strain) and saprophyte 457, isolated from cabbage seed.

[2] Test was conducted with heated cells.

[3] The number of strains of which the complete cell wall was well stained.

[4] The number of strains of which the cell wall was only partially stained.

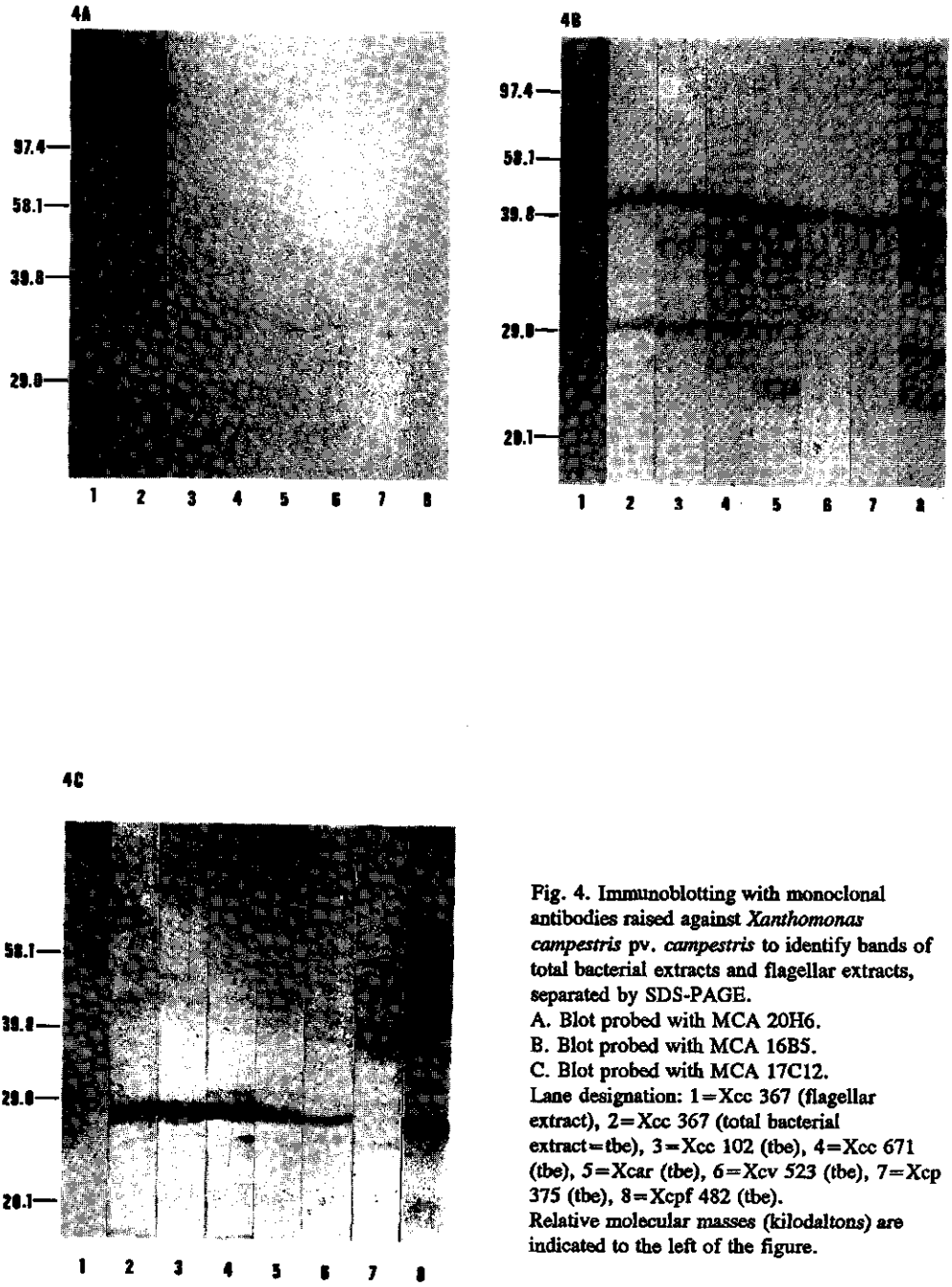

Fig. 4. Immunoblotting with monoclonal antibodies raised against *Xanthomonas campestris* pv. *campestris* to identify bands of total bacterial extracts and flagellar extracts, separated by SDS-PAGE.
A. Blot probed with MCA 20H6.
B. Blot probed with MCA 16B5.
C. Blot probed with MCA 17C12.
Lane designation: 1=Xcc 367 (flagellar extract), 2=Xcc 367 (total bacterial extract=tbe), 3=Xcc 102 (tbe), 4=Xcc 671 (tbe), 5=Xcar (tbe), 6=Xcv 523 (tbe), 7=Xcp 375 (tbe), 8=Xcpf 482 (tbe).
Relative molecular masses (kilodaltons) are indicated to the left of the figure.

minor band was sometimes noted at 29 kDa (Xcc 367, Xcc 102, Xcc 671, Xcar and Xcpf 482) and 23 kDa (Xcar and Xcpf 482). MCA 17C12 (fig. 4C) reacted with a major band at 29 kDa of Xcc 367, Xcc 102, Xcc 671, Xcar, Xcv 523 and Xcpf 482 (resp. lanes 2-6 and 8). For Xcp 375 no reaction was found with MCA 17C12. A reaction with a 29 kDa band was not detected in flagellar extracts of Xcc strain 367 (lane 1, fig. 4C), but was detected in cell envelope extracts (not shown).

Discussion

The specificity of PCAs in IF and immunoblotting

Xcc strains 367, 102 and 365 reacted variably with the PCAs in IF. Also in SDS-PAGE and immunoblotting differences in reactions of PCAs 94, 111 and 113 with the LPS of these strains were found. This indicated that LPS patterns may be unique for strains of Xcc as is known for *Pseudomonas* strains (De Weger et al., 1987) and that the LPS may contain epitopes, that are unique for certain Xcc strains or group of Xcc strains.

The preparation and characterization of flagellar extracts used for immunization

For immunization of mice, crude extracts were used as immunogens because low-antibody titres were obtained in mice immunized with purified LPS. The same phenomenon was found by De Boer and McNaughton (1987) for the LPS from *Erwinia carotovora* subsp. *atroseptica*, serogroup I. Flagellar extracts contained both the LPS and protein bands which could possibly act as specific immunogens (fig. 3).

SDS-PAGE profiles of flagellar extracts (fig. 2) showed two major protein bands, viz. a 44 kDa band, that is believed to contain the heat modifiable major outer membrane protein A of Xcc (Minsavage and Schaad, 1983) and a 58 kDa band which may contain the protein subunits of the flagella (flagellin) of Xcc. This, however, needs further confirmation.

The production and selection of MCAs produced against Xcc

In this study MCAs were produced against flagellar extracts of Xcc strain 367. Immunization with cell envelope extracts of Xcc strains were also done but they did not yield hybridomas, that produced antibodies reacting strongly with Xcc strains. Moreover, many cross-reactions were obtained in EIA and DBI. Therefore, we did not proceed with cell envelope extracts as immunogens.

Although the MCAs discussed in this study were produced against flagellar extracts, we did not find good staining of flagella in IF. Some explanations may be given. Possibly, in initial selections using EIA the antigen may have been denatured, which could have led to not selecting suitable clones. As EIA was found to be more sensitive than indirect IF for detecting positive hybridoma supernatants (Jones and Van Vuurde, 1989), we used EIA for initial selections. Still, we did not obtain clones producing antibodies reacting with the 44 or 58 kDA bands (fig. 2). Since motility was present in all strains tested prior to preparation of flagellar extracts, which indicates presence of flagella, we believe that the protein antigens used were poorly immunogenic, possibly due to the presence of highly repeating determinants (e.g. flagellin) which predominantly yields low affinity IgM antibodies (Roitt, 1988). We aimed at selecting high affinity IgG antibodies.

The results presented in table 3 show that when selecting for suitable clones, great differences appear between serological tests, as was found for MCAs 16B5 and 17C12. A possible explanation is that the exposition of the epitope was variable and strain dependent in EIA and IF. In DBI the exposition of the epitope is probably less variable and not strain-dependent, which could make DBI therefore more suited for selection of antibodies against typical antigens.

The reaction of MCAs was studied in a selection procedure with cells boiled for 15 min at 100 °C to diminish non-specific background reaction of Xcc strains (table 3), possibly due to endogenous phosphatase activity (El-Sharkawy and Huisingh, 1971). Background reactions occasionally occurred for strains of Xcc and Xcp but not for strains of Xcv and all strains of other bacterial species tested. Boiling extracts did not significantly change the reactivity of the antibodies indicated in table 3 compared to earlier selections with whole, unboiled cells in the DBI.

The characterization and specificity of MCAs produced to Xcc

In this study, MCAs (20H6, 18G12, 2F4, 10C5) were obtained, that reacted specifically with the LPS of a group of Xcc strains and some strains of closely related pathovars of *X. campestris*. Reactions with non-xanthomonads were not detected. None of our LPS-specific MCAs were pathovar-specific or reacted with all Xcc strains. Similar results were found for Xcc by Alvarez et al. (1985). The results obtained with MCAs confirmed observations done in immunoblotting experiments with PCAs and cell envelope extracts (fig. 1), i.e. LPS may contain epitopes unique for a group of Xcc strains and related strains of other pathovars. LPS-specific antibodies for plant pathogenic bacteria were produced earlier by Benedict et al. (1989) for *X. c.* pv. *oryzae* and pv. *oryzicola* and by De Boer and McNaughton (1987) for *Erwinia carotovora* subsp. *atroseptica*, serogroup I. Benedict et al. (1990) even found MCAs specific for the LPS of all strains of *X. c.* pv. *begoniae* and pv. *pelargonii*.

MCAs (17C12 and 16B5) were obtained that reacted with proteins of Xcc and of related pathovars of *X. campestris*. They did not cross-react with non-xanthomonads. The nature of the homologous epitopes is not known. However, the major 39 kDa and 29 kDa band to which MCA 16B5 and MCA 17C12, respectively, reacted on immunoblots, could be the major protein bands of outer membrane extracts identified by Minsavage and Schaad (1983) and Laakso et al. (1990) in SDS-PAGE. Since the 39 kDa and 29 kDa bands were present in cell envelope extracts, and MCAs 16B5 and 17C12 generally gave good reactions in IF, it is assumed that these proteins are (thermo-stable) surface antigens often present in outer membranes of Xcc. A reaction of MCA 17C12 with the 29 kDa band was not found in flagellar extracts of Xcc strain 367 (fig. 4C) although this band was shown to be present in flagellar extracts (fig. 3, weak reaction of PCA 113 with this band in extracts of Xcc 367 in immunoblotting). This was probably due to the low concentration of this band in the flagellar extract. A reaction of MCA 17C12 with flagellar extracts was obtained in DBI indicating that the homologous antigen was present in the flagellar extract.

At some dilutions of the PCAs cross-reactions with Xcv as well as with Xca are possible. The MCAs discussed here are also able to cross-react with Xcv or Xca. Also

Schaad (1978), Alvarez et al. (1985), and Alvarez and Lou (1985) found a serological relationship of Xcc with Xcv and/or Xca. Since Xcv is host-specific for tomato, this cross-reaction will not be a problem in the detection of Xcc in crucifer seeds. Xca can affect crucifers and therefore the significance of the cross-reaction with this bacterium needs to be investigated. To assess the problems occurring with MCAs in the detection of Xcc in crucifer seeds, serological assays should be done in parallel with dilution-plating assays. This will provide information on the specificity of the MCAs and the sensitivity of serological tests with MCAs when applied to plant extracts.

We conclude that the monoclonal and polyclonal antibodies discussed in this study, may be used to differentiate and identify (groups of) Xcc strains in several serological tests. Studies are now in progress to investigate the application of the monoclonal and polyclonal antibodies for the detection of Xcc in crucifer seeds.

Acknowledgements

Thanks are due to P.S. van der Zouwen and M. Poppelier for excellent technical assistance. The critical comments of prof. dr ir J. Dekker and dr R. Bino are gratefully acknowledged.

Thanks are also due to IPO-DLO (Research Institute for Plant Protection) for kindly providing polyclonal antisera. The cooperation of IPO-DLO, PD (Plant Protection Service, Wageningen), and drs C. van Henten, T.L. Kuan, D.L. Pinnow, B. Rat, N. Thaveechai, F. Vinther and P.H. Williams in providing cultures is also gratefully acknowledged.

References

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

Alvarez, A.M., Benedict, A.A. & Mizumoto, C.Y., 1985. Identification of

xanthomonads and grouping of strains of *Xanthomonas campestris* pv. *campestris* with monoclonal antibodies. Phytopathology 75: 722-728.

Benedict, A.A., Alvarez, A.M., Berestecky, J., Imanaka, W., Mizumoto, C.Y., Pollard, L.W., Mew, T.W., & Gonzalez, C.F., 1989. Pathovar-specific monoclonal antibodies for *Xanthomonas campestris* pv. *oryzae* and for *Xanthomonas campestris* pv. *oryzicola*. Phytopathology 79: 322-328.

Benedict, A.A., Alvarez, A.M. & Pollard, L.W., 1990. Pathovar-specific antigens of *Xanthomonas campestris* pv. *begoniae* and X. *campestris* pv. *pelargonii* detected with monoclonal antibodies. Applied and Environmental Microbiology 56: 572-574.

Boonekamp, P.M., Pomp, H. & Gussenhoven, G.C., 1990. Production and characterization of monoclonal antibodies to potato virus A. Journal of Phytopathology 128: 112-124.

Clark, M.F. & Adams, A.N., 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology 34: 475-483.

De Boer, S.H. & McNaughton, M.E., 1987. Monoclonal antibodies to the lipopolysaccharide of *Erwinia carotovora* subsp. *atroseptica* serogroup I. Phytopathology 77: 828-832.

De Weger, L.A., Jann, B., Jann, K. & Lugtenberg, B., 1987. Lipopolysaccharides of *Pseudomonas* spp. that stimulate plant growth: composition and use for strain identification. Journal of Bacteriology 169: 1441-1446.

El-Sharkawy, T.A. & Huisingh D., 1971. Differentiation among *Xanthomonas* species by polyacrylamide gel electrophoresis of soluble proteins. Journal of General Microbiology 68: 155-165.

Franken, A.A.J.M. & Van Vuurde, J.W.L., 1990. Problems and new approaches in the use of serology for seed-borne bacteria. Seed Science and Technology 18: 415-426.

Haaheim, L.R., Kleppe, G. & Sutherland, I.W., 1989. Monoclonal antibodies reacting with the exopolysaccharide xanthan from *Xanthomonas campestris*. Journal of General Microbiology 135: 605-612.

Jones, J.B. & Van Vuurde, J.W.L., 1989. Monoclonal antibodies (MAs) against

Xanthomonas campestris pv. *begoniae* (XCB) and *pelargonii* (XCP). Phytopathology 79: 1178 (Abstr.).

Kiredjian, M., Holmes, B., Kersters, K., Guilvout, I. & De Ley, J., 1986. *Alcaligenes piechaudii*, a new species from human clinical specimens and the environment. International Journal of Systematic Bacteriology 36: 282-287.

Laakso, T., Ojanen, T., Helander, I.M., Karjalainen, R., Korhonen, T.K. & Haahtela, K., 1990. Comparison of outer membrane proteins and lipopolysaccharides of *Xanthomonas campestris* pathovars. Proceedings of the 7th International Conference on Plant Pathogenic Bacteria: 149-154.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Martin, J.H. & Savage, D.C., 1985. Purification and characterization of flagella from *Roseburia cecicola*, an obligately anaerobic bacterium. Journal of General Microbiology 131: 2075-2078.

Minsavage, G.V. & Schaad, N.W., 1983. Characterization of membrane proteins of *Xanthomonas campestris* pv. *campestris*. Phytopathology 73: 747-755.

Roitt, I., 1988. Essential Immunology. Blackwell Scientific Publications, London, pp. 286.

Schaad, N.W., 1978. Use of direct and indirect immunofluorescence tests for identification of *Xanthomonas campestris* pv. *campestris*. Phytopathology 68: 249-252.

Tsai, C.M. & Frasch, C.E., 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Analytical Biochemistry 119: 115-119.

Van Vuurde, J.W.L., Van den Bovenkamp, G.W. & Birnbaum, Y., 1983. Immunofluorescence microscopy and enzyme-linked immunosorbent assay as potential routine tests for the detection of *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* in bean seed. Seed Science and Technology 11: 547-559.

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

Yuen, G.Y., Alvarez, A.M., Benedict, A.A., & Trotter, K.J., 1987. Use of monoclonal

antibodies to monitor the dissemination of *Xanthomonas campestris* pv. *campestris*. Phytopathology 77: 366-370.

Chapter 5. Application of polyclonal and monoclonal antibodies for the detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds using immunofluorescence microscopy

Application of polyclonal and monoclonal antibodies for the detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds using immunofluorescence microscopy

Abstract

Polyclonal and monoclonal antibodies (PCAs and MCAs) were tested for the detection of *Xanthomonas campestris* pv. *campestris* (Xcc) in cabbage seeds using immunofluorescence microscopy (IF). It was concluded that PCA 94, MCAs 20H6, 2F4, 18G12 and a mixture of MCAs 20H6, 18G12, 2F4 and 16B5 could be used to detect Xcc in seed extracts when 5 min and 2.5 h shaking of seeds are used as extraction methods. The reliability of confirming suspect colonies with MCAs and PCA 94 in IF depended in part on the seed lot tested and the antibody used. Some virulent Xcc strains derived from seed lots, did not react with MCAs 10C5, 2F4, 18G12, 17C12 and 16B5. On the other hand, saprophytic isolates obtained from one seed lot cross-reacted with MCA 17C12 and to a lesser extent with MCAs 2F4, 18G12 and PCA 94. No relationship was found between IF-reactions of Xcc strains using MCAs and reactions of Xcc strains in pathogenicity testing. Xcc and *X. c.* pv. *amoraciae* (Xca) could in general not be distinguished on the basis of reactions with MCAs and PCAs. Also in pathogenicity tests Xcc and Xca were hard to distinguish.

Additional keywords: pathogenicity testing, virulence, dilution-plating, YDC-agar, NSCA, NSCAA, nutrient starch medium, antibiotics.

Introduction

Xanthomonas campestris pv. *campestris* (Xcc), the causal agent of black rot in crucifers, is a seed-transmitted plant-pathogenic bacterium (Richardson, 1990). The use of disease-

free seed is an important way to prevent establishment and spread of this pathogen. For detection of Xcc in seeds, techniques should be both specific and sensitive. Serological assays potentially meet these requirements. Schaad (1978) and Schaad and Donaldson (1980) used immunofluorescence microscopy (IF) with polyclonal antibodies (PCAs) to identify colonies and to detect Xcc in cabbage seed extracts, respectively. However, cell counts in IF were not reported, although they could give information on the specificity and sensitivity of IF. Alvarez and Lou (1985) used ELISA with PCAs to detect Xcc in leaf disk samples from field-grown cabbage. Yuen et al. (1987) used ELISA with monoclonal antibodies (MCAs) to detect and to differentiate strains of Xcc isolated from cabbage black rot lesions collected from the field. Franken et al. (1992) recently evaluated MCAs for identification of Xcc strains in IF, an enzyme immunoassay and a dot-blot immunoassay.

The aim of the present study was to investigate the application of MCAs, produced against Xcc (Franken et al., 1992) as compared to a specific polyclonal antiserum (PCA 94), for detection of Xcc in crucifer seeds with IF. IF was chosen because this test was considered to be the most sensitive method for the detection of seed-borne bacteria (Franken and Van Vuurde, 1990). For direct detection of Xcc in seed extracts, cell counts in IF were used for comparison of MCAs with PCA 94.

Materials and methods

Monoclonal and polyclonal antibodies

All antibodies used in this study were described by Franken et al. (1992). MCAs were purified using ammonium sulphate precipitation or cation-exchange chromatography. PCA 94, MCA 20H6 (5 mg ml^{-1}), MCA 16B5 (5 mg ml^{-1}), MCA 2F4 (5 mg ml^{-1}), MCA 18G12 (5 mg ml^{-1}), 10C5 (2.5 mg ml^{-1}) and 17C12 (5 mg ml^{-1}) were used in dilutions of 1:100, 1:100, 1:100, 1:30, 1:30, 1:10 and 1:100, respectively. The mixture of MCA (Mix-MCA) was prepared by mixing equal volume aliquots of MCA 20H6 (5 mg ml^{-1}), MCA 16B5 (5 mg ml^{-1}), MCA 2F4 (5 mg ml^{-1}) and MCA 18G12 (5 mg ml^{-1}) and used at a 1:100 dilution.

Bacterial strains

All cultures used in this study are listed in table 1.

Table 1. Bacterial strains used in immunofluorescence microscopy and pathogenicity testing for *Xanthomonas campestris* pv. *campestris*.

Bacteria	Strain designation	Received from [1]
Bacillus subtilis	Bsub 6633	5
Erwinia herbicola	Eher 732, Eher 1062, Eher 1063,	4
	Eher 83	1
E. carotovora subsp. *carotovora*	Ecar 139	1
Pseudomonas marginalis	Pmar 86/1118	4
P. viridiflava	Pvir 540	1
P. cichorii	Pcich 478	4
Xanthomonas campestris pv. *amoraciae*	Xca 373, Xca 374	1
X. c. pv. *campestris*	Xcc 102, Xcc 364, Xcc 367, Xcc 671	1
	Xcc 8-6, Xcc 9-48, Xcc 9-4, Xcc 9-30N,	2
	Xcc 8-19-1	2

[1] 1=Research Institute for Plant Protection (IPO-DLO), Wageningen, the Netherlands; 2= Author; 3= D.L. Pinnow, Georgia, USA; 4=Plant Protection Service, Wageningen, the Netherlands; 5= American Type Culture Collection, Maryland, USA

Immunofluorescence microscopy

Naturally contaminated seed lots from various origins were tested with IF (10 000 seeds per seed lot). To each sample of 10 000 seeds 100 ml of saline (0.85% NaCl) was added. All samples were shaken for 5 min and 2.5 h at room temperature. Twenty μl per subsample (undiluted and 10x diluted) was fixed onto multitest slides (8 or 5 mm diameter). Indirect IF was done as described by Van Vuurde et al. (1983). At least 25 microscope fields were counted (field coefficient = 18, objective magnification x63, internal magnification x1.25 or x1.00, ocular magnification x10). Cell counts were converted to the number of fluorescent cells per ml. For analysis of variance, all cell counts

were transformed to logarithms of fluorescent cells per ml (log cells per ml). To include zero readings one cell per ml was added to each cell count.

Plating

Plating was done on NSCA (nutrient starch cycloheximide agar; Schaad and Donaldson, 1980) and NSCAA (NSCA with the addition of nitrofurantoin and vancomycin; Randhawa and Schaad, 1984) as described by Franken et al. (1991). Suspect colonies from NSCA and NSCAA were confirmed on YDC (yeast extract-dextrose-calcium carbonate agar; Schaad, 1988), in IF using PCA 94 and MCAs, and pathogenicity testing. A seed lot was considered to be infested with Xcc, when YDC-positive (yellow mucoid) colonies produced typical black discolouration of the veins in the pathogenicity test, described by Schaad (1982).

Pathogenicity tests

Cultures were grown for 24-48 h on a growth factor medium or on YDC agar. For all pathogenicity tests, cultivars 'Septa' (*Brassica oleracea* var. *capitata*) and 'Tardis' (*Brassica oleracea* var. *gemmifera*) were used.

The pathogenicity test, published by Schaad (1982), was used as a reference (pathogenicity test 1). For this pathogenicity test, light milky cell suspensions (10^7-10^8 cells ml^{-1}) were made in distilled water and inoculations took place immediately after preparing the suspensions. At least two plants of each cultivar were inoculated per isolate. Plants were incubated at 22-25 °C.

Pathogenicity test 2 was a modification of a pathogenicity test suggested by B. Schrijver, Bejo Zaden B.V. (personal communication). In this test, seeds free of Xcc and fungal pathogens and with germination above 90% were treated with AAtiram (50% a.i. thiram), Rovral (50% a.i. iprodione) and Benlate (50% a.i. benomyl), according to standard procedures prescribed by the manufacturers, to avoid possible interference by fungi after inoculation. Twenty-five seeds per filter paper disk were germinated according to the Rules of the International Seed Testing Association (ISTA, 1985). After 3-4 days, seed coats were removed from the seedlings and cotyledons were dipped for 5 min into

cell suspensions of pure cultures or YDC-positive colonies. Contact of the cell suspensions with the filter paper was avoided. After inoculation, filter papers were put onto humid silversand in a small tray. Incubation took place at 22-25 °C, and a minimum of 12 hours light per day was given. Symptoms appeared within 4-10 days. Typical Xcc-symptoms were initially papery brown to black localised lesions on margins of infected cotyledons and at the end of the middle vein of cotyledons (often V-shaped). With proceeding infection, cotyledons and hypocotyls often became water-soaked. Cotyledons finally collapsed. Controls were bacterial strains of *Erwinia herbicola, E. carotovora* subsp. *carotovora, Pseudomonas marginalis, P. viridiflava, P. cichorii* and *Bacillus subtilis*.

Results

Screening seed lots with IF using polyclonal and monoclonal antibodies

The results of screening seed lots with IF using MCAs and PCA 94 are summarized in table 2. The cell counts in IF (expressed as log cells per ml) varied significantly with (i) the seed lot tested, (ii) the extraction method and (iii) the antibodies used ($P < 0.001$). Although in most cases, more fluorescent cells were found after 2.5 h than after 5 min shaking, exceptions were found for some combinations of seed lots and antibodies. For example, seed lot B189 tested with MCA 17C12, seed lot B190 tested with PCA 94 and seed lot B214 tested with MCA 18G12 yielded higher cell counts after 5 min than after 2.5 h shaking.

PCA 94, MCAs 20H6, 2F4, 18G12 and the Mix-MCA generally gave high cell counts, whereas cell counts obtained with MCAs 17C12, 10C5 and 16B5 were generally lower or nearly zero. However, exceptions were found, e.g. when using PCA 94 and 5 min shaking a negative result was obtained for seed lot B189 whereas high cell counts were obtained with MCA 17C12. The same was valid when comparing cell counts obtained with resp. MCA 20H6 and MCA 16B5 for seed lot B188 using 5 min shaking.

Table 2. Cell counts in immunofluorescence microscopy using monoclonal and polyclonal antibodies, produced against *Xanthomonas campestris* pv. *campestris*.

Seed lot	Antibodies	Extraction method (log cells per ml)[1]	
		5 min shaking	2.5 h shaking
B188	PCA 94	2.00	2.44
	MCA 20H6	0.00	0.00
	MCA 2F4	0.00	0.00
	MCA 18G12	0.00	0.00
	Mix-MCA	0.00	1.00
	MCA 10C5	0.00	0.00
	MCA 17C12	0.00	1.50
	MCA 16B5	1.71	0.00
B189	PCA 94	0.00	4.28
	MCA 20H6	3.88	4.47
	MCA 2F4	3.84	4.87
	MCA 18G12	3.97	4.05
	Mix-MCA	3.91	4.81
	MCA 10C5	3.62	4.27
	MCA 17C12	4.24	1.09
	MCA 16B5	0.00	1.00
B190	PCA 94	2.70	1.45
	MCA 20H6	1.20	2.65
	MCA 2F4	2.59	1.20
	MCA 18G12	2.59	1.00
	Mix-MCA	0.85	1.27
	MCA 10C5	1.59	2.20
	MCA 17C12	0.00	0.00
	MCA 16B5	0.00	0.00
B196	PCA 94	2.65	1.24
	MCA 20H6	2.00	1.15
	MCA 2F4	0.00	1.45
	MCA 18G12	0.00	1.39
	Mix-MCA	0.00	1.27
	MCA 10C5	0.00	0.00
	MCA 17C12	2.18	1.30
	MCA 16B5	0.00	0.00
B197	PCA 94	4.54	6.17
	MCA 20H6	4.87	5.70
	MCA 2F4	4.46	6.05
	MCA 18G12	4.26	5.94
	Mix-MCA	4.39	5.65
	MCA 10C5	3.90	6.03
	MCA 17C12	0.00	2.79
	MCA 16B5	1.09	0.00

(continued on next page)

Table 2 (continued).

Seed lot	Antibodies	Extraction method (log cells per ml)[1]	
		5 min shaking	2.5 h shaking
B209	PCA 94	4.84	5.71
	MCA 20H6	4.53	5.89
	MCA 2F4	4.23	6.00
	MCA 18G12	4.36	5.84
	Mix-MCA	4.28	5.69
	MCA 10C5	3.54	5.38
	MCA 17C12	0.00	3.93
	MCA 16B5	0.00	0.00
B213	PCA 94	4.69	4.84
	MCA 20H6	4.09	4.83
	MCA 2F4	4.39	4.70
	MCA 18G12	4.51	5.10
	Mix-MCA	4.55	5.39
	MCA 10C5	1.09	4.52
	MCA 17C12	3.07	3.50
	MCA 16B5	0.00	0.00
B214	PCA 94	1.15	3.02
	MCA 20H6	1.00	0.00
	MCA 2F4	4.95	3.54
	MCA 18G12	4.69	2.98
	Mix-MCA	3.10	1.75
	MCA 10C5	0.00	4.21
	MCA 17C12	0.00	0.00
	MCA 16B5	0.00	0.00
B215	PCA 94	1.50	0.00
	MCA 20H6	1.09	2.55
	MCA 2F4	0.00	1.20
	MCA 18G12	0.00	1.20
	Mix-MCA	0.85	1.27
	MCA 10C5	0.00	0.00
	MCA 17C12	1.15	1.15
	MCA 16B5	0.00	0.00
B216	PCA 94	4.48	4.21
	MCA 20H6	4.40	4.25
	MCA 2F4	2.83	4.18
	MCA 18G12	3.66	4.23
	Mix-MCA	3.55	4.21
	MCA 10C5	3.49	3.83
	MCA 17C12	1.33	1.39
	MCA 16B5	0.00	0.00

[1]Means of 2 replications; standard error of differences of means = 0.905.

For seed lots B197, B209 and B213 usually high cell counts were obtained (cell counts expressed as log cells per ml varied generally between 4 and 6). This, however, also depended on the antibodies used.

In general, the fluorescence intensity of cells in IF-slides stained with MCA 10C5 was inferior to the fluorescence intensity of cells stained with other MCAs and PCA 94. On the other hand, the fluorescence intensity of cells stained by MCA 20H6 was superior to that of cells stained by other MCAs and PCA 94 (not shown).

In the plating assays corresponding to the IF tests from table 2, seed lots B189, B197, B213 using 5 min and 2.5 h shaking and seed lot B209 using 2.5 h shaking were found positive (infested).

The use of polyclonal and monoclonal antibodies for identification of suspect colonies

Suspect colonies were transferred from NSCA and NSCAA to YDC. PCA 94 and MCAs were used to identify colonies from YDC. MCAs 2F4, 18G12, 10C5 and 17C12 reacted with lower numbers of colonies in IF than PCA 94 and MCA 20H6 (table 3). MCA 16B5 generally gave negative IF-results for all colonies tested. For most seed lots using PCA 94, MCA 20H6 and the Mix-MCA a good correlation was found between YDC results and confirmations by IF, e.g. for PCA 94 with seed lots B189, B197, B209 and B213, MCA 20H6 with seed lots B189, B197, B209 and B213. The Mix-MCA reacted generally in a similar way as PCA 94 and MCA 20H6, except for seed lot B213, for which some 'false-negative' colonies (i.e. Xcc colonies not reacting with the antibodies) were found. For seed lot B214 some YDC-negative colonies (saprophytic isolates) were found to cross-react with PCA 94, MCAs 2F4, 18G12, 17C12 and the Mix-MCA in IF. The results of the pathogenicity tests correlated well with the YDC-results and IF-confirmations using MCA 20H6 and PCA 94; nearly one hundred per cent of the YDC-positive and IF-positive (using PCA 94 and MCA 20H6) results were positive in the pathogenicity tests.

Table 3. Confirmation of colonies, derived from crucifer seed lots, with YDC-medium, IF and pathogenicity testing.

Seed lot	Number of[1] colonies tested	YDC result[2] (number of positive colonies)	IF result[3] (number of positive colonies)							Pathogenicity test[4] (number of positive colonies)
			PCA 94	MCA						
				20H6	2F4	18G12	Mix	10C5	17C12	16B5
B188	13	0	0	0	0	0	0	0	0	0
B189	12	6	6	6	6	6	6	6	1	0	6
B190	9	0	0	0	0	0	0	0	0	0	0
B196	3	0	0	0	0	0	0	0	0	0	0
B197	15	10	10	10	9	9	10	8	4	0	10
B209	15	7	6	7	5	4	6	3	2	0	7
B213	20	16	16	16	9	8	12	14	3	1	16
B214	20	0	2	0	2	1	1	0	4	0	0
B215	16	0	0	0	0	0	0	0	0	0	0
B216	5	0	0	0	0	0	0	0	0	0	0

[1] The number of colonies tested is the number of colonies transferred from NSCA or NSCAA to YDC.
[2] The number of positive colonies is the number of colonies which showed typical colony morphology and pigment production on YDC.
[3] The number of positive colonies is the number of colonies from YDC which showed good staining of the whole cell wall in IF.
[4] The number of positive colonies is the number of colonies which showed symptoms 'typical' for *Xanthomonas campestris* pv. *campestris*.

Comparison of IF-reactions to reactions in pathogenicity tests

To investigate a possible correlation between reactions in IF and the degree of virulence, several known reference Xcc strains were tested in IF and pathogenicity test 1 and 2. Table 4 shows the reactions of some reference Xcc strains with MCAs in IF and their reaction on plants. Low virulent strains 9-30 N and 8-6 and virulent strains 102 and 671 did not react with MCA 20H6. MCA 16B5 and 17C12 generally reacted equally well with low virulent strains 9-30 N and 8-6 and highly virulent strains such as Xcc 367 and Xcc 364. MCA 2F4 did not react with low virulent strains 9-30 N and 8-6 and virulent strain 671. MCA 18G12 reacted with strains 8-6 (low virulence) and 367 (high virulence) but not with 9-30 N (low virulence) and 9-4 (moderate virulence). MCA 10C5 did not react with strains 9-30 N and 8-6 (low virulence) but did also not react with strain 671 (moderate virulence). Neither of the negative control strains produced symptoms in these pathogenicity tests, nor did they react with any of the antibodies tested.

In a separate experiment differences between Xcc strains 364 and 367, and Xca strains 373 and 374 were evaluated. These strains were tested in IF and inoculated on plants of cultivars 'Septa' and 'Tardis' using pathogenicity test 1 and 2. In IF, Xca 373 reacted with all the MCAs tested. Xca 374 did not react with MCA 10C5, but reacted weakly with the other MCAs. Xcc 364 failed to react with MCA 20H6, but reacted with all other MCAs. Xcc 367 and Xca 373 reacted identically with the MCAs in IF (results not shown).

In pathogenicity test 1, inoculation with Xca strains 373 and 374 occasionally resulted in appearance of localized brown to black discolouration of one vein and chlorotic leaf spots on cultivars 'Tardis' and 'Septa' (no systemic symptoms). Xcc strains 364 and 367 produced on both cultivars systemic black discolouration of the veins, followed by appearance of leaf lesions and desiccation of the lesion tissue. In pathogenicity test 2 on cultivar 'Septa', 96%±3.7% (25 seedlings per isolate, two replicates per isolates) of the cotyledons were speckled (necrotic spots) after infection by Xca strains 373 and 374. For Xcc strains 364 and 367, 14%±18.2% of the cotyledons was speckled. Also on cultivar 'Tardis' speckled cotyledons were found for Xca strains 373 and 374 (41%±24.8%), whereas no speckled cotyledons were found for Xcc 364 and 367. However, all plants of

Table 4. Relationship between reactions in IF using several antibodies and the degree of virulence on cultivars 'Septa' and 'Tardis'.

Strains	Reaction of several strains in pathogenicity tests[1]				Reactions with monoclonal antibodies in IF[1]					
	pathogenicity test 1		pathogenicity test 2		20H6	16B5	17C12	2F4	18G12	10C5
	Septa	Tardis	Septa	Tardis						
Xcc 102	+	+	+	+	-	++	+	±	+	±
Xcc 8-19-1	++	++	++	++	++	-	±	±	±	+
Xcc 9-30 N	±/-	±/-	±	±	-	+	+	-	-	-
Xcc 8-6	±/-	±/-	±	±	-	+	+	-	+	-
Xcc 9-4	+	+	++	±	+	+	+	-	-	+
Xcc 671	++	+	+	+	-	+	+	-	+	-
Xcc 367	++	++	++	+	++	+	+	+	+	+
Xcc 364	+	+	+	+	-	+	+	+	+	+
Negative controls	-	-	-	-	-	-	-	-	-	-

[1] ++ = very strong reaction; + = strong reaction; ± = weak reaction; - = no reaction.

both cultivars also showed "typical Xcc-symptoms" in pathogenicity test 2 after inoculation with Xcc and Xca strains, viz. initially papery brown to black localised lesions on margins of infected cotyledons and at the end of the middle vein of cotyledons (often V-shaped). With proceeding infections, cotyledons and hypocotyls often became water-soaked. Cotyledons finally collapsed.

In both pathogenicity tests the reactions on cultivar 'Tardis' were somewhat weaker than on cultivar 'Septa'. Also the incubation time was longer on cultivar 'Tardis' than on cultivar 'Septa'.

Discussion

Screening seed lots with IF using polyclonal and monoclonal antibodies

Since IF is considered to be the most sensitive serological assay (Franken and Van Vuurde, 1990), this test was used to screen seed lots. The results presented in this study (table 2) generally agreed well with results obtained earlier using antibodies from hybridoma supernatants (Franken, 1990). Cell counts depended on the antiserum, extraction method, and the seed lot used. The reasons for these interactions are not completely clear. Some explanations may be given. First, the expression of epitopes may differ between seed lots. This would explain why e.g. for MCA 16B5, that reacted with more Xcc strains (pure cultures) in IF than any other MCA used in this study (Franken, 1990; Franken et al., 1992), low cell counts were obtained. Second, seed lots may contain serologically different strains, of which some are predominant in a specific seed lot and do not react with e.g. MCA 16B5. In addition, phase variation ('a phenomenon which involves a spontaneous switch from the synthesis of one given cell-surface component or structure to another, antigenically distinct form of that component or structure' (Singleton and Sainsbury, 1987)) may be present within a Xcc strain. Third, saprophytes and seed components may affect the growth and serological reactions of Xcc. Strong evidence for one of these explanations has, however, not been found, yet.

As shown in table 2, the Mix-MCA gave almost equal cell counts as PCA 94, MCA 20H6, MCA 2F4 and MCA 18G12, although this varied per seed lot and extraction method. No additional effect was noted by using a mixture of antibodies compared to using one MCA. However, the result may depend on the specific dilutions and antibodies used.

The dilution-plating results obtained in the present study did not completely correspond with results from earlier studies (Franken, 1990; Franken et al., 1991). In the latter studies seed lots B188, B190, B196, B214, B215 and B216 were found positive in dilution-plating whereas they were found negative in this study. This may be explained by the decrease in viability of Xcc cells during storage of the seed lots as is known for *Pseudomonas syringae* pv. *phaseolicola* (Taylor et al., 1979). However, the presence of

dead or 'dormant' cells does not need to have special implications for the characteristics of IF as such, since dead or 'dormant' cells can still be detected with IF.

On the basis of indexing a limited number of seed lots, it is concluded that PCA 94, MCA 20H6, MCA 2F4, MCA 18G12 and the Mix-MCA may be used to detect Xcc in seed extracts with IF. A prerequisite is that seed lots should be screened by using two extraction methods, viz. 5 min and 2.5 h shaking to minimize the risk that seed lots are incorrectly identified as being 'healthy'. To assess the risk of false-negative and false-positive reactions in more detail, and the occurrence of interactions between seed lots and extraction methods, IF is now used in combination with dilution-plating for routine indexing of crucifer seed lots using 5 min and 2.5 h shaking. When using both dilution-plating and IF, additional information on the health status of a seed lot will be obtained. Moreover, information on the sensitivity of IF as compared to dilution-plating will become available, which will help to decide whether IF-positive seed lots which are negative in dilution-plating should be considered infested or not. Studies to investigate correlations between IF and dilution-plating are now being conducted with more seed lots.

The use of polyclonal and monoclonal antibodies for identification of suspect colonies by IF

When using PCA 94, MCA 20H6 and the Mix-MCA in IF generally good correlations were obtained between IF and YDC confirmation tests. However, when confirming 315 colonies derived from dilution-plating assays done earlier with seed lots B188, B189, B213 and B216 (Franken, 1990), YDC-positive and IF-positive results corresponded for 98.2 and 80% when using PCA 94 and MCA 20H6, respectively. Especially for seed lots B213 and B216 not all YDC-positive results could be confirmed by MCA 20H6 whereas positive reactions with PCA 94 almost completely corresponded with YDC-positive colonies (A.A.J.M. Franken, unpublished). Other antibodies were not tested in this particular case. The IF-tests on colonies with MCAs confirmed to a large extent the results with respect to the direct detection of Xcc in seed extracts. However, by using MCAs 2F4, 18G12, 16B5, 10C5 and 17C12 for confirmation of suspect colonies the risk of 'false-negative' reactions is larger than when using PCA 94 and MCA 20H6.

It should be noted that growth rate differences between strains, derived from the same seed lots, were frequently observed on NSCA, NSCAA, YDC and the growth factor medium. These observations suggest that colonies vary in extracellular polysaccharide production and may contain alterations in the cell envelope (Cadmus et al., 1976; Kamoun and Kado, 1990; Kidby et al., 1977), that may seriously influence the reliability of the serological reactions. This emphasizes the fact that more information is needed to establish a possible relationship between physiological conditions and serological reactions of Xcc strains.

The question may also arise whether strains change in their reactions with MCAs in IF or in pathogenicity tests after prolonged storage on slants or after repeated passage through plants. This, however, has not been noted during this or other studies (Yuen et al., 1987).

Reactions in IF compared to reactions in pathogenicity testing

No correlation was found between reactions of Xcc strains on plants of two susceptible cultivars and reactions with MCAs in IF. For an exact analysis of the correlation between reactions with MCAs in IF and the degree of virulence, however, pathogenicity testing on different and more diverse cultivars of several *Brassica* species is necessary.

Differentiation of Xca and Xcc strains was not possible on the basis of reaction with the PCA 94 and the MCAs in IF. The cross-reaction of Xca strains with antibodies raised against Xcc strains was reported earlier and mentioned as a potential problem in detection and identification of Xcc strains (Alvarez and Lou, 1985; Franken, 1990; Franken et al., 1992). Options to be considered for distinguishing Xca from Xcc strains are e.g., sodium dodecyl sulphate-polyacrylamide gel electrophoresis and fatty-acid profiling, isozyme analysis and DNA hybridization methods. For seed testing and phytosanitary certification, however, pathogenicity testing will often remain necessary for 100% reliability. As suggested by Machmud and Black (1985) Xca causes leaf spot, which is not caused by Xcc. They also found that disease severity as a result of Xca infection varied among cabbage cultivars and that optimum temperatures for Xcc and Xca may differ. In the present study, Xca gave considerably more necrotic spots on cotyledons in pathogenicity test 2

than Xcc. In pathogenicity test 1 symptoms caused by Xcc were more severe and tended to be more systemic than symptoms caused by Xca. The differences noted are in part quantitative differences, and depend in part on the cell concentration applied and cultivar used. For confirmation of colonies by pathogenicity testing, it is therefore difficult to distinguish Xca from Xcc, especially when weakly virulent Xcc strains are isolated from seed lots. Few reports with information about seed infection by Xca are available, whereas numerous reports are available on seed infection with Xcc (Machmud and Black, 1985; Richardson, 1990). The role and significance of Xca in causing a major disease of crucifers is therefore not clear. Therefore, it is advisable to consider seed lots infested when colonies are found positive on YDC and in pathogenicity testing, regardless of whether individual colonies should be regarded as Xca or Xcc.

Acknowledgements

Thanks are due to P.S. van der Zouwen, Y. Birnbaum, J. van Hateren and M. Schippers for technical assistance. The help of seed companies in providing seed lots is gratefully acknowledged. Thanks are also due to prof. dr ir J. Dekker, dr R. Bino and dr C.J. Langerak for critically reading the manuscript.

References

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

Cadmus, M.C., Rogovin, S.P., Burton, K.A., Pittsley, J.E., Knutson, C.A. & Jeanes, A., 1976. Colonial variation in *Xanthomonas campestris* NRRL B-1459 and characterization of the polysaccharide from a variant strain. Canadian Journal of Microbiology 22: 942-948.

Franken, A.A.J.M., 1990. Production, characterization and application of monoclonal antibodies for detecting *Xanthomonas campestris* pv. *campestris*. In: A. Schots (Ed.),

Proceedings of the symposium Perspectives for monoclonal antibodies in agriculture, p. 27-36. PUDOC, Wageningen, the Netherlands.

Franken, A.A.J.M. & Van Vuurde, J.W.L., 1990. Problems and new approaches in the use of serology for seed-borne bacteria. Seed Science and Technology 18: 415-426.

Franken, A.A.J.M., Zilverentant, J.F., Boonekamp, P.M. & Schots, A., 1992. Specificity of polyclonal and monoclonal antibodies for the identification of *Xanthomonas campestris* pv. *campestris*. Netherlands Journal of Plant Pathology 98: 81-94.

Franken, A.A.J.M., Van Zeijl, C., Van Bilsen, J.G.P.M., Neuvel, A., De Vogel, R., Van Wingerden, Y., Birnbaum, Y.E., Van Hateren, J. & Van der Zouwen, P.S., 1991. Evaluation of a plating assay for *Xanthomonas campestris* pv. *campestris*. Seed Science and Technology 19: 215-226.

ISTA, 1985. International rules for seed testing. Seed Science and Technology 13: 520 pp.

Kamoun, S. & Kado, C.I., 1990. Phenotypic switching affecting chemotaxis, xanthan production, and virulence in *Xanthomonas campestris*. Applied and Environmental Microbiology 56: 3855-3860.

Kidby, D., Sandford, P., Herman, A. & Cadmus, M., 1977. Maintenance procedures for the curtailment of genetic instability: *Xanthomonas campestris* NRRL B-1459. Applied and Environmental Microbiology 33: 840-845.

Machmud, M. & Black, L.L., 1985. A *Xanthomonas* leaf spot of crucifers caused by *Xanthomonas campestris* pv. *amoraciae*. Contributions of the Central Research Institute for Food Crops, Bogor 74: 1-13.

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

Richardson, M.J., 1990. An annotated list of seed-borne diseases (fourth edition), ISTA Handbook on seed health testing, Section 1.1. International Seed Testing Association, Zürich, Switzerland.

Schaad, N.W., 1978. Use of direct and indirect immunofluorescence tests for identification of *Xanthomonas campestris* pv. *campestris*. Phytopathology 68: 249-252.

Schaad, N.W., 1982. *Xanthomonas campestris* pv. *campestris*. Working sheet no. 50, ISTA Handbook on seed health testing, Section 2. International Seed Testing Association, Zürich, Switzerland.

Schaad, N.W., 1988. Laboratory guide for identification of plant pathogenic bacteria, p. 3. The American Phytopathological Society, St. Paul, Minnesota, USA.

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

Singleton, P. & Sainsbury, S., 1987. Dictionary of microbiology and molecular biology (second edition). John Wiley & Sons, Chichester.

Taylor, J.D., Dudley, C.L. & Presly, L., 1979. Studies of halo-blight seed infection and disease transmission in dwarf beans. Annals of Applied Biology 93: 267-277.

Van Vuurde, J.W.L., Van den Bovenkamp, G.W. & Birnbaum, Y.E., 1983. Immunofluorescence microscopy and enzyme-linked immunosorbent assay as potential routine tests for the detection of *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* in bean seed. Seed Science and Technology 11: 547-559.

Yuen, G.Y., Alvarez, A.M., Benedict, A.A., & Trotter, K.J., 1987. Use of monoclonal antibodies to monitor the dissemination of *Xanthomonas campestris* pv. *campestris*. Phytopathology 77:366-370.

Chapter 6. Comparison of immunofluorescence microscopy and dilution-plating for the detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds

Comparison of immunofluorescence microscopy and dilution-plating for the detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds

Abstract

The correlation between immunofluorescence microscopy (IF) and dilution-plating on nutrient starch cycloheximide agar (NSCA) or NSCA with the addition of nitrofurantoin and vancomycin (NSCAA) was studied for the detection of *Xanthomonas campestris* pv. *campestris* (Xcc) in crucifer seeds. When checking 50 µl of the seed extract in IF, IF and dilution-plating gave corresponding results (both positive or negative) for 45.4-56.4% of the samples tested. No differences were observed in this respect between tests using a polyclonal antiserum (PCA 94) and replicate tests using monoclonal antibodies (MCA 20H6). When 20 µl of the seed extract in IF was checked, 67.3-71.3% of the samples tested were both positive or negative with dilution-plating and IF. IF negative and dilution-plating positive samples were found for 0.0-7.3% of all samples tested. The percentage of IF positive and dilution-plating negative samples ranged from 26.7-29.2 (20 µl seed extract checked) to 41.8-47.3% (50 µl seed extract checked). Generally, the probability of isolating Xcc increased with increasing numbers of fluorescent cells found in IF. Above 10 000 cells per ml the probability of isolating Xcc ranged from 57.1-81.8%. Increasing the extraction time from 5 min to 2.5 h shaking showed no significant increase of the number of samples found positive in IF and dilution-plating. However, when using both 5 min and 2.5 h shaking as compared to 5 min shaking only, more samples can be found positive in IF (1.0-14.5%) and dilution-plating (3.0-18.5 %). Examining 1 µl instead of 50 µl of the sample smear, would increase the correspondence between IF and dilution-plating results up to minimally 69.1% (MCA 20H6). However, the risk of false-negative results in IF as compared to dilution-plating would also increase.

Additional keywords: correlation, test evaluation, extraction method, media, serology, seed-borne bacteria, *Pseudomonas syringae* pv. *phaseolicola*.

Introduction

In many laboratories immunofluorescence microscopy (IF) or dilution-plating are both used to detect seed-borne bacteria (Franken and Van Vuurde, 1990). However, little is known about the correlation between results obtained by both techniques. So far, only for *Pseudomonas syringae* pv. *phaseolicola* (Psp, the causal agent of halo blight) in bean seeds and *Xanthomonas campestris* pv. *campestris* (Xcc, the causal agent of black rot) in crucifer seeds, an analysis of the correspondence and discrepancy between results obtained by both tests has been made to a certain extent (respectively Van Vuurde et al. (1991) and Schaad (1982, 1983b)). Van Vuurde et al. (1991), using polyclonal antibodies in IF, compared test data of 710 samples and showed that IF was able to indicate the potential risk of halo blight for bean seed lots which were negative in dilution-plating. They demonstrated that the probability of isolating Psp increases with increasing numbers of fluorescent cells in IF. A 'grey' area of discrepancy between IF and dilution-plating was found for 17.6% of all seed samples tested. In their case the grey area included the IF positive and dilution-plating negative samples. IF negative samples were never found positive in dilution-plating in earlier experiments (Van Vuurde et al., 1983). Schaad and Donaldson (1980), using polyclonal antibodies in IF, found a grey area of discrepancy between IF and dilution-plating for 1.5% (n=273) and 3.3% (n=121) of the crucifer seed lots tested for Xcc in two separate experiments. The grey area included dilution-plating positive and IF negative samples as well as dilution-plating negative and IF positive samples. Schaad and Donaldson (1980) recommended that Xcc should be detected in crucifer seeds by assaying with dilution-plating and IF staining tests. Schaad (1982) found a grey area for 17.2% of all seed lots tested for Xcc. This grey area included dilution-plating positive and IF negative samples as well as dilution-plating negative and IF positive samples. Schaad (1983b) found a grey area of discrepancy of 50-60%, although only just 10 samples were tested. Schaad (1982, 1983b) using polyclonal antibodies in IF,

dissuaded IF for seed certification because of lack of correlation between IF and dilution-plating on one hand, and IF and field incidence of black rot on the other hand. However, a detailed analysis of the relation between the number of fluorescent cells found in IF and the probability of isolating Xcc was not made.

The aim of the present study was to analyse in more detail the correlation between IF, using monoclonal and polyclonal antibodies, and dilution-plating for detecting Xcc in crucifer seeds.

Materials and methods

Seed lots

Naturally contaminated and 'healthy' seed lots were obtained from different parts of the world. Crucifer seed lots with low to high Xcc contamination levels (known from earlier tests and field trials) were chosen for experiment 1. Seed lots from various origins which in earlier tests gave variable results in plating assays on NSCAA (see below) or which had high saprophyte numbers were chosen for experiment 2. For experiment 3 trade seed lots were tested in a seed certification program without any foreknowledge of the level of Xcc contamination or the origin of the seed lot.

Extraction method

Xcc was extracted from seed lots as described by Franken et al. (1991). In short, each cabbage seed lot tested for Xcc consisted of 10 000 or 2000 seeds. Per 1000 seeds, 10 ml of saline (0.85% NaCl) was added and seed lots were shaken for 5 min or 2.5 h at room temperature. After 5 min or 2.5 h shaking, liquid was taken for IF as well as dilution-plating.

Plating

Plating for Xcc was done on a nutrient starch cycloheximide agar (NSCA; Schaad and Donaldson, 1980) and a nutrient starch cycloheximide agar with the addition of nitrofurantoin and vancomycin (NSCAA; Randhawa and Schaad, 1984), with some

modifications described by Franken et al. (1991). Suspected colonies were tested for their identity on yeast-extract-dextrose-calcium carbonate agar (YDC) and in a pathogenicity test (Schaad, 1989).

Antibodies and IF

Monoclonal antibody 20H6 (MCA 20H6) and polyclonal antiserum 94 (PCA 94) were used in IF for detecting Xcc. PCA 94 and MCA 20H6 were described and tested for specificity by Franken et al. (1992). Indirect IF was done as described by Van Vuurde et al. (1983). All IF-slides were examined by epifluorescence microscopy using a 500-1000x magnification, a field coefficient (i) of 18 and a tube coefficient (R or K) of 1.00-1.25. Fluorescein-isothiocyanate (FITC) was used for IF as a fluorochrome.

Statistical analysis of the data

Analysis of variance was done using the statistical program Genstat (Rothamsted Experimental Station). χ^2-tests were used for testing differences between frequencies as described by Siegel (1956). All effects and differences were tested for significance at 95% probability level.

Results

IF and dilution-plating were both used to index seed lots for presence of Xcc. The samples tested were classified as found positive (infested) or negative (not infested) by IF and dilution-plating. The results are summarized in table 1. When using MCA 20H6 in experiments 1 (50 μl examined), 2 (50 μl examined) and 3 (20 μl examined), respectively 52.8%-56.4%, 52.7% and 67.3-71.3% of all seed samples were found both positive or both negative in dilution-plating and IF. Testing the same samples with PCA 94 in experiment 1, the results obtained with IF and dilution-plating corresponded for 45.4-56.4%. When using MCA 20H6 in experiments 1, 2 and 3, 0.0-1.9% of all samples tested was found to be negative in IF but positive in dilution-plating. With PCA 94 a percentage of 1.8-7.3 was found in experiment 1. In general, MCA 20H6 and PCA 94

Table 1. Comparison of immunofluorescence microscopy (IF) and dilution-plating for detecting *Xanthomonas campestris* pv. *campestris* in crucifer seeds.

Study[1]	Extraction method	Number of positive samples in dilution-plating		Number of negative samples in dilution-plating		Percentage of both IF and dilution-plating positive or negative samples
		IF +	IF -	IF +	IF -	
Experiment 1						
PCA 94	5 min shaking	17 (30.9%)	4 (7.3%)	26 (47.3%)	8 (14.5%)	45.4%
	2.5 h shaking	24 (43.7%)	1 (1.8%)	23 (41.8%)	7 (12.7%)	56.4%
MCA 20H6	5 min shaking	20 (36.4%)	1 (1.8%)	25 (45.4%)	9 (16.4%)	52.8%
	2.5 h shaking	24 (43.7%)	1 (1.8%)	23 (41.8%)	7 (12.7%)	56.4%
Experiment 2						
MCA 20H6	5 min shaking	26 (13.9%)	3 (1.6%)	86 (45.7%)	73 (38.8%)	52.7%
Experiment 3						
MCA 20H6	5 min shaking	18 (17.8%)	0 (0.0%)	33 (32.7%)	50 (49.5%)	67.3%
	2.5 h shaking	13 (12.9%)	2 (1.9%)	27 (26.7%)	59 (58.4%)	71.3%

[1]In experiment 1 and 2 50 μl of the sample was examined in IF; in experiment 3 20 μl.

did not differ with respect to cell counts in IF. Cell counts were, however, depended on the sample tested ($P<0.05$). A great part of the samples tested in experiment 1 and 2 were IF positive and dilution-plating negative. In experiment 3 a great part of the samples tested were IF negative and dilution-plating negative.

In experiment 1 and 3 two extraction methods were compared, viz. 5 min and 2.5 h shaking. None of the differences were significant, as tested by a χ^2-test.

A more detailed analysis was made of the relation between IF and plating results. The results presented in table 2 show the number of positive seed samples in dilution-plating in relation to the number of fluorescent cells per ml in IF. Table 2 shows that generally more samples were found positive in dilution-plating when more fluorescent cells were found in IF. For example, when using MCA 20H6 and 2.5 h shaking in experiment 1 23.5% of the samples classified in IF-class 1.3-2.0 were positive in dilution-plating. Of the samples classified in IF-class 4-6, 70.0% was found to be positive in dilution-plating. Some exceptional results were found, e.g. in experiment 3 more samples were found positive in dilution-plating in IF-class 1.7-2.0 than in IF-class 2.0-4.0. The coefficient of correlation r between log cells per ml in IF and log cfu per ml of Xcc in dilution-plating was always within the range of 0.5-0.6.

In general, no differences were found between PCA 94 and MCA 20H6 in the distribution of the percentage of plating-positive samples over the IF-classes in experiment 1 ($P=0.234$). Also no significant differences were found between 5 min and 2.5 h shaking in experiment 1 and 3 with respect to the percentage of positive isolations in the IF-classes ($P=0.621$).

Most of the cabbage seed samples tested in experiment 1 were classified in the IF-classes (log fluorescent cells per ml) ranging from 2.0-4.0 and 4.0-6.0. In experiment 2 most samples were classified in the IF-class ranging from < 1.3 and 2.0-4.0. In experiment 3 most samples were classified in the IF-class < 1.7. A relatively high percentage (12.5-33.3%) of the samples classified in the IF-class <1.3 in experiment 1 was dilution-plating positive whereas in experiment 2 and 3 the percentages were relatively low (3.9 and 0.0-3.3%, respectively).

Table 2. The probability of isolating *Xanthomonas campestris* pv. *campestris* on NSCA and NSCAA in different classes with increasing numbers of fluorescent cells found in immunofluorescence microscopy (IF).

Experiment no.	Antibody	Extraction method	Number of samples positive in dilution-plating/Number of samples tested per IF class, based on 50 µl smear				
			< 1.3[1]	1.3-2.0	2.0-4.0	4.0-6.0	>6.0
1	PCA 94	5 min shaking	4/12 (33.3)[2]	4/15 (26.7)	4/13 (30.8)	9/15 (60.0)	0/0 (-)
		2.5 h shaking	1/8 (12.5)	0/5 (0.0)	8/20 (40.0)	15/21 (71.4)	1/1 (100.0)
	MCA 20H6	5 min shaking	1/10 (10.0)	2/14 (14.3)	8/19 (42.1)	9/11 (81.8)	1/1 (100.0)
		2.5 h shaking	1/8 (12.5)	4/17 (23.5)	6/10 (60.0)	14/20 (70.0)	0/0 (-)
2	MCA 20H6	5 min shaking	3/76 (3.9)	4/39 (10.3)	9/53 (16.7)	12/19 (63.1)	1/1 (100.0)
			Number of samples positive in dilution-plating/Number of samples tested per IF class, based on 20 µl smear				
			< 1.7[1]	1.7-2.0	2.0-4.0	4.0-6.0	>6.0
3	MCA 20H6	5 min shaking	0/50 (0.0)	5/16 (31.2)	1/20 (5.0)	12/15 (80.0)	0/0 (-)
		2.5 h shaking	2/61 (3.3)	3/8 (37.5)	2/17 (11.8)	8/14 (57.1)	0/1 (0.0)

[1]IF-classes are expressed as logarithms to base 10 of the number of fluorescent cells per ml in IF; < 1.3 and < 1.7 equals to a contamination less than 20 (50 µl of the sample smear examined) or 50 (20 µl of the sample smear examined) cells per ml, respectively ('IF negative' samples).
[2]Between brackets: the percentage of positive samples per IF-class.

Discussion

In the present study IF and dilution-plating were used to index seed lots for presence of Xcc. IF had the same characteristics in this study (for Xcc) as for other seed-borne bacteria such as Psp (Van Vuurde et al., 1991). More samples were found positive in IF than in dilution-plating for MCA 20H6 as well as PCA 94 (table 1). Also Schaad (1982, 1983a, 1983b) reported for Xcc that IF could find more seed lots positive than dilution-plating. Several explanations for these results can be given. First, IF allows to detect fluorescent cells in undiluted extracts amongst high numbers of saprophytes whereas in dilution-plating a ten or hundred times dilution from the extract is sometimes needed to reduce the interference of saprophytes. Second, dead or weak cells may be detected in IF which cannot be detected in dilution-plating. Third, cross-reactions may have caused false-positive reactions in IF. However, the occurrence of cross-reactions is less likely, since a specificity-tested antiserum was used in the present study (Franken et al., 1992; Franken, 1992) and other studies cited here (Schaad and Donaldson, 1980; Schaad, 1983a, 1983b). No cross-reacting bacteria were isolated in this study. Fourth, seed lots may have contained chemical substances produced by the seeds or other micro-organisms which may have prevented growth of the pathogen.

In spite of the high sensitivity of IF, 0-7.3% of all samples were found to be IF negative and dilution-plating positive in this study (table 1). Schaad and Donaldson (1980) and Schaad (1982) found respectively 0.4%-5.4% of all seed lots to be both IF negative and dilution-plating positive (table 3).

The correlation between IF and dilution-plating for detecting Xcc in crucifer seed lots was compared with results reported by Schaad and Donaldson (1980) and Schaad (1982, 1983a, 1983b) (table 3). The correlation between IF and dilution-plating was less for the seed lots tested in this study than for seed lots tested by Schaad and Donaldson (1980) and Schaad (1982). This could be due to differences in the volume of the sample smear examined. Schaad (1983a, 1983b) refers to Schaad and Donaldson (1980), who examined 1 μl. This means that the maximum sensitivity would be 1000 fluorescent cells per ml. In experiments 1 and 2 (50 μl of the sample smear examined) the maximum sensitivity

Table 3. Comparison of immunofluorescence microscopy (IF) and dilution-plating results from several studies with regard to the detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds.

Study	Extraction method	Volume of smear examined in IF	Percentage of samples that gave corresponding results in both IF and dilution-plating (both assays positive or negative)	Calculated percentage[1] of both IF and dilution-plating positive or negative samples, when 1 µl of sample smear would have been examined	Calculated percentage[1] of both IF negative and dilution-plating positive samples, when 1 µl of sample smear would have been examined
experiment 1, MCA 20H6	5 min	50 µl	52.8% (n=55)	> 70.9%	> 5.5%
	2.5 h	50 µl	56.4% (n=55)	> 72.7%	> 9.1%
experiment 2, MCA 20H6	5 min	50 µl	52.7% (n=188)	> 69.1%	> 3.7%
experiment 3, MCA 20H6	5 min	20 µl	67.3% (n=101)	> 73.3%	> 5.0%
	2.5 h	20 µl	71.3% (n=101)	> 73.3%	> 5.0%
Other studies					
Schaad and Donaldson (1980)	1 min	1 µl	96.7% (n=121)	96.7%	3.3%
			98.5% (n=273)	98.5%	0.4%
Schaad (1982)	not stated[2]	not stated[2]	82.8% (n=423)	unknown[2]	unknown[2] (5.4%)[3]
Schaad (1983b)	1 min	1 µl	40% (n=10)	40%	0.0%
			50% (n=12)	50%	0.0%

[1] Calculated by regarding samples classified in IF-class 1.3-2.0 and 1.7-2.0 (Table 2) as 'IF negative'.
[2] No detailed description of the IF-procedure was given.
[3] Between brackets: the percentage of IF negative and dilution-plating positive samples given by the reference.

would be 20 fluorescent cells per ml. Therefore, using the data mentioned in table 2 for calculating table 3, this would mean that the IF positive samples given in IF-class 1.3-2.0 and a part of IF-class 2-4 (table 2) would not have been found IF-positive by the method used by Schaad and Donaldson (1980). Table 3 shows that the correlation between IF and dilution-plating would be higher if 1 μl (minimally 69.1% correspondence between IF and dilution-plating results) instead of 50 μl (52.7-56.4% correspondence between IF and dilution-plating results) of the bacterial smear would have been examined (table 3). This approximates to the data given by Schaad (1982) and Schaad and Donaldson (1980) and is supported by the fact that in experiment 3, in which 20 μl was examined, a higher correlation (67.3-71.3%) between IF and dilution-plating was found. By checking 1 μl instead of 50 μl, the detection threshold of IF will decrease and approximate to the detection threshold of dilution-plating. However, in that case the percentage of IF negative and dilution-plating positive samples will increase up to a level above 3.7% (table 3). The fact that Schaad (1983b) observed a relatively poor correlation between IF and dilution-plating may have been due to the small number of seed lots tested.

The differences between results obtained in this study and the studies by Schaad (1982, 1983b) and Schaad and Donaldson (1980), may also in part be explained by the possibility that seed lots differed with respect to the levels of saprophytes and treatments (e.g. storage) undergone prior to seed testing. Also different extraction methods have been used. However, Schaad (1989) stated that short washes at 25 °C are generally satisfactory to isolate the pathogen from the seed.

The comparison of 5 min and 2.5 h shaking showed no differences in number of samples found positive in IF and dilution-plating (table 1). This confirms the data presented by Franken et al. (1991). Also the percentage of positive isolations per IF-class did not differ between 5 min and 2.5 h shaking (table 2). However, using both 5 min and 2.5 h shaking of seed lots instead of 5 min shaking only would have resulted in finding more samples infested. In this study, an extra 3.0-18.5% of the samples tested would have been found positive in dilution-plating when both 5 min and 2.5 h shaking would have been used instead of 5 min shaking only (data not shown). In IF, an extra 1.0%-14.5% of the samples tested would have been found positive when both 5 min and 2.5 h

shaking would have been used instead of 5 min shaking only (data not shown). This suggests that the use of two extraction methods enhances the probability of detecting Xcc in various crucifer seed lots.

To improve the correlation between IF and dilution-plating, the plating assays may be improved by using more selective media with a high recovery of the target bacterium, such as CS20ABN medium (Chang et al., 1991). This medium was reported to detect more infested seed lots than the NSCA, NSCAA and FS medium.

To decide which test (IF or dilution-plating) is preferable for detecting Xcc in seed lots some aspects of the disease and the test should be considered. When the disease is considered to be a disease with a quarantine status, generally, a prediction of 'health' is required. When a certain tolerance for a disease is accepted, a prediction of 'disease' is required. Data derived directly or calculated from the reports by Van Vuurde et al. (1991) for Psp and Schaad (1983b) for Xcc show that IF has a relatively high predictive value of a negative result ('health'). Dilution-plating has a relatively high predictive value of a positive result ('disease'). When a prediction of 'health' of a seed lot is required, IF would give a better prediction than dilution-plating. When a prediction of 'disease' is required, dilution-plating would give a better prediction than IF. Thus, when dilution-plating positive results are obtained, the seed lot should be regarded as 'being infested'. When both IF and dilution-plating negative results are found, the seed lot should be regarded as 'being healthy' or infested at a level below the tolerance level. When dilution-plating negative results and IF positive results are found, 'risk classes' should be used based on the number of saprophytes isolated on the plates, the number of fluorescent cells found in IF, and field studies under optimum 'disease conditions'. Such a classification scheme is now used for indexing bean seeds for Psp in the Netherlands (J.W.L. van Vuurde, unpublished).

It should, however, be noted that, as reported by Sheppard et al. (1986), the predictive values of a positive result and negative result vary with the prevalence of the disease (defined here as the amount of infested seeds per seed lot). At a low prevalence of a disease, given a specified diagnostic sensitivity and specificity, the percentage of 'false-positive' results will be much higher than at a high prevalence of the disease.

Acknowledgements

The author is grateful to prof. dr ir J. Dekker, drs R. Bino and C.J. Langerak, and Mrs P.S. van der Zouwen for critically reading the manuscript. Thanks are due to ir J. van Bilsen, Bejo Seeds B.V. and M. Hooftman, Nederlandse Algemene Keuringsdienst voor Siergewassen (NAKS), for providing data for comparison and for comments on the manuscript.

References

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

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.

Franken, A.A.J.M. & Van Vuurde, J.W.L., 1990. Problems and new approaches in the use of serology for seed-borne bacteria. Seed Science and Technology 18: 415-426.

Franken, A.A.J.M., Zilverentant, J.F., Boonekamp, P.M. & Schots, A., 1992. Specificity of polyclonal and monoclonal antibodies for the identification of *Xanthomonas campestris* pv. *campestris*. Netherlands Journal of Plant Pathology 98: 81-94.

Franken, A.A.J.M., Van Zeijl, C., Van Bilsen, J.G.P.M., Neuvel, A., De Vogel, R., Van Wingerden, Y., Birnbaum, Y., Van Hateren, J. & Van der Zouwen, P.S., 1991. Evaluation of a plating assay for *Xanthomonas campestris* pv. *campestris*. Seed Science and Technology 19: 215-226.

Randhawa, P.S. & Schaad, N.W., 1984. Selective isolation of *Xanthomonas campestris* pv. *campestris*. Phytopathology 74: 268-272.

Schaad, N.W., 1982. Detection of bacterial plant pathogens. Plant Disease 66: 885-890.

Schaad, N.W., 1983a. Detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds. Seed Science and Technology 11: 573-578.

Schaad, N.W., 1983b. Correlation of laboratory assays for seedborne bacteria with disease development. Seed Science and Technology 11: 877-883.

Schaad, N.W., 1989. Detection of *Xanthomonas campestris* pv. *campestris* in crucifers. In: A.W. Saettler, N.W. Schaad & D.A. Roth (Eds), Detection of Bacteria in Seed and other Planting Material. The American Phytopathological Society, St. Paul, Minnesota, p. 68-75.

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

Sheppard, J.W., Wright, P.F. & DeSavigny, D.H., 1986. Methods for the evaluation of EIA tests for use in the detection of seed-borne diseases. Seed Science and Technology 14: 49-59.

Siegel, S., 1956. Nonparametric statistics for the behavioral sciences. McGraw-Hill Book Company, Inc., Tokyo, Japan, 312 pp..

Van Vuurde, J.W.L., Franken, A.A.J.M., Birnbaum, Y. & Jochems, G., 1991. Characteristics of immunofluorescence microscopy and of dilution-plating to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed lots and for risk assessment of field incidence of halo blight. Netherlands Journal of Plant Pathology 97: 233-244.

Van Vuurde, J.W.L., Van den Bovenkamp, G.W. & Birnbaum, Y., 1983. Immunofluorescence microscopy and enzyme-linked immunosorbent assay as potential routine tests for the detection of *Pseudomonas phaseolicola* and *Xanthomonas phaseoli* in bean seeds. Seed Science and Technology 11: 547-559.

Chapter 7. Problems and new approaches in the use of serology for seed-borne bacteria

Problems and new approaches in the use of serology for seed-borne bacteria

Abstract

In routine indexing of seed samples for plant pathogenic bacteria, detection methods should have high sensitivity and specificity. Also the possibilities to standardise and automate, as well as the rapidity of a detection method are important features with regard to the application of the method to large quantities of seed. Serological tests potentially meet these specific requirements. However, the lack of commercially available high quality antisera and standardisation in the application of seed testing procedures are serious drawbacks. Descriptions of standardised methods, centralisation of antiserum production and international training courses are needed to overcome these drawbacks. A disadvantage of the commonly used serological techniques in seed testing, e.g. immunofluorescence micropscopy (IF) and enzyme-linked immunosorbent assay (ELISA), is the lack of distinction between viable and dormant or dead cells. This often results in a 'grey' area of discrepancy between results of serological and traditional isolation assays. New techniques such as immonufluorescence colony staining, direct double diffusion in dilution plates, immunosorbent immunofluorescence and immuno-isolation combine isolation and serological techniques and have the potential to overcome this 'grey' area.

Introduction

Serological tests for plant pathogenic bacteria have been known since 1918 (Schaad, 1979). Since that time many research workers have recognised the ability of serological tests to provide quick and reliable detection or identification. During the last decades the interest in serological tests and the importance of serology in detecting or identifying plant pathogenic bacteria, including seed-borne bacteria, has greatly increased. One of the reasons for the increasing interest is the development of ways to obtain better antisera,

e.g. the development of the hybridoma technology to produce monoclonal antibodies and of tests to characterise and isolate specific antigens of bacteria (Alvarez, Benedict and Mizumoto, 1985; De Boer and McNaughton, 1987). Although the production of monoclonal antisera is mentioned here, the application of monoclonal antibodies is still limited. Polyclonal antisera are still widely used in seed bacteriology.

The most commonly used, also with the bacteriology working group of the International Seed Testing Association (ISTA), serological tests for detecting and identifying seed-borne bacteria are:

- immunofluorescence microscopy (Akerman, Zutra, Volcani and Henis, 1973; Coleno, 1968; Coleno, Trigalet and Digat, 1976; ISTA, 1981, 1982, 1984; Malin, Roth and Belden, 1983; Schaad, 1978; Trigalet and Bidaud, 1978; Trigalet and Rat, 1976; Van Vuurde, Van den Bovenkamp and Birnbaum, 1983),
- enzyme-linked immunosorbent assays (Alvarez and Lou, 1985; Barzic and Trigalet, 1982; Elango and Lozano, 1980; Erwin and Khan, 1987; Lamka, McGee, Hill and Braun, 1987; Weaver and Guthrie, 1978),
- Ouchterlony double diffusion (Guthrie, 1968; Guthrie, Huber and Fenwick, 1965; Tanii, Takakuwa, Baba and Takita, 1976; Trujillo and Saettler, 1979) and
- agglutination (Guthrie et al., 1965; ISTA, 1981, 1982; Tanii et al., 1976; Taylor, 1970).

Drawbacks

Serological tests are used for several reasons. First, serological tests are suited for routine application because of their rapidity and easy application to large amounts of (seed) samples (Schaad, 1979; Trigalet, Samson and Coleno, 1978). Second, some of the serological tests, such as immunofluorescence microscopy (IF), are very sensitive (Schaad, 1979, 1982; Van Vuurde, 1987a, b); others are very specific such as Ouchterlony double diffusion (Schaad, 1979). However, the sensitivity partly depends on the working procedure used for the test. For example, the sensitivity of IF increases with increasing numbers of observed microscope fields. The sensitivity of this test also depends on other parts in the working procedure, such as sample extraction and slide

preparation, and on the quality of the microscope, used in this test. Thus, standardisation of working procedures of serological tests is very important (Schaad, 1979; Trigalet et al., 1978). Some serological tests are more standardisable than others, e.g. an enzyme-linked immunosorbent assay (ELISA) can be easily automated and is therefore usually easily standardised and also suited for screening large numbers of seed samples (Sheppard, 1983; Sheppard, Wright and DeSavigny, 1986). IF is somewhat less standardisable, especially due to the, sometimes, subjective reading of IF-slides.

For nearly all serological tests different working procedures are known and used by different laboratories all over the world. Even when standard descriptions of working procedures are available, they might be interpreted differently. When working procedures used in different laboratories are completely the same, test results may vary between laboratories due to the use of different antisera.

The degree of specificity of antisera may be another drawback in the use of serological tests (Trigalet et al., 1978). Antisera may vary in quality. Some antisera may give cross-reactions, others may be too specific because they only detect one or a few serogroups of the pathogen population. Few or no laboratories exist which produce and supply antisera of a guaranteed quality in adequate quantities for seed-borne bacteria, which limits the application of serological tests in seed bacteriology.

Some of the drawbacks, mentioned here, may be solved by intensively comparative testing as happens within the Bacteriology working group of ISTA, aiming at optimising and standardising working procedures for each test. Further, training courses (e.g. workshops) for interested laboratories will increase the knowledge about serological tests and will make it easier for laboratories to start with serology. The problem of the supply of antisera can be solved by centralising antiserum production and quality testing at centres which have the experience and knowledge to produce and test antisera for a specific pathogen (Schaad, 1982).

As the specificity of an antiserum depends on the specificity of the antigen injected (Schaad, 1979), more research is needed to find typical antigenic determinants for various species of plant pathogenic bacteria. Typical antigenic determinants may be the LPS (De Boer and McNaughton, 1987) and the membrane proteins (Minsavage and Schaad, 1983;

Thaveechai and Schaad, 1986a, 1986b).

The testing of antisera should aim at a guarantee of quality, with respect to reactions with a standard collection of representative pathogen strains, saprophytes and strains of other pathogens and should take place with the appropriate test (Schaad, 1979).

Traditional application of serological tests

Since isolation of pathogen colonies is often necessary for 100% reliability and because one needs pure cultures for other tests or research, serological tests are often applied as screening tests on seed leachates before isolation or identification tests on colonies after isolation. To decide which test can be used for a specific purpose, some properties of these tests have to be known. Some general properties of serological tests for seed-borne bacteria are summarised in table 1. Generally, IF has the highest consistent sensitivity of the tests mentioned (Van Vuurde, 1987b). Therefore, IF is often used as a screening method to rapidly distinguish non-infected seed samples from potentially infected seed samples (Malin et al., 1983; Van Vuurde et al., 1983). After IF test results are known, potentially infected seed samples (i.e. IF-positive samples) are often plated in order to isolate viable pathogen cells (Van Vuurde et al., 1983). Because the other mentioned serological tests lack a comparable sensitivity, these tests are generally not suited for use as screening tests. However, each of these tests, including IF, is suited as an identification test. ELISA has the advantage of its great potential for standardisation and automation which is of importance for routine seed health testing laboratories (Sheppard et al., 1986). Agglutination is recommended for its simplicity and rapidity (Schaad, 1979). Ouchterlony double diffusion (ODD) has a high specificity, meaning that ODD has the ability to differentiate closely related antigens and can therefore be used to identify specific strains or serogroups of the pathogen (Schaad, 1979). IF has the advantage that besides the antigen-antibody reaction also the cell morphology of the bacterium can be checked.

At the Netherlands Government Seed Testing Station, IF is used to routinely index bean (*Phaseolus vulgaris* L.) samples for the pathogen *Pseudomonas syringae* pv. *phaseolicola* (Van Vuurde et al., 1983). When fluorescent cells have been found in IF for a particular

Table 1. Some important properties of serological techniques commonly used in the detection of seed-borne bacteria.

	ELISA	Ouchterlony double diffusion	IF	slide-agglutination
Analytical specificity***	2-3*	1	2	3
Analytical sensitivity***	3**	4	2	3
Potential standardisation	1*	1	2	1-2
Potential applicability to large numbers	1*	2-3	2	2

* 1 = very high; 2 = high; 3 = moderate; 4 = low

** 1 = very high = 10^2-10^3 cells/ml; 2 = high = 10^3-10^5 cells/ml; 3 = moderate = 10^5-10^7 cells/ml; 4 = low > 10^7 cells/ml

*** The terminology of Sheppard et al. (1986) is used.

(sub)sample, leachate of this IF-positive sample is plated on a medium. IF-negative samples are not plated. Suspected colonies are confirmed by IF and a pathogenicity test. There is a so-called grey area between the IF screening method and the plating method; IF-positive samples are somtimes found to be negative in dilution-plating. One explanation is the variable and sometimes low sensitivity of isolation, due to large amounts of saprophytic bacteria, which can overgrow the pathogen or inhibit the pathogen from producing typical colonies on the plates (Van Vuurde, 1987b). In these cases it is hard to obtain a test result, since it is also known for sure whether viable pathogen cells (fluorescent cells) are present in the seed sample.

To solve this problem one could use (semi-)selective media, which have the advantage of inhibiting part of the saprophytes from forming colonies. However, these media are sometimes not adequately selective (many saprophytes will still grow on the media) or may be too selective. For seed samples with very low infection levels and/or 'weak' or 'damaged' pathogen cells which need resuscitation, selective media may lead to false negative results when the pathogen cells are all killed or to much lower test values when a part of the pathogen cell population is killed. Moreover, growth of the pathogen is

sometimes retarded on selective media which means one has to wait longer for a final test result.

Several formats of ODD, ELISA and IF have been described. Also incubation conditions, buffers and equipment may differ considerably between laboratories. For IF and ODD, several critical steps and materials have been discussed for seed-borne bacteria and standard procedures were suggested (ISTA, 1988). Some critical factors of ELISA have been evaluated for seed-borne diseases, although no standard procedure was suggested (Sheppard et al., 1986). Recommended procedures should be outlined. However, it seems unrealistic to prescribe a universal standard for each serological test, since each sample - antibody combination may need specific modifications with regard to incubation conditions and materials.

New techniques

Assays such as immunofluorescence colony staining, direct double diffusion and immuno-isolation tests combine advantages of dilution-plating and serology (Van Vuurde, 1987b; Van Vuurde and Van Henten, 1983; Van Vuurde, Ruissen and Vruggink, 1987). They can be used to overcome the problems of traditional serological tests and are presently developed at RPvZ and IPO. These techniques should be considered as new approaches towards serology and are not yet fully developed for use on a routine basis in seed bacteriology.

Immunofluorescence colony staining

Research started at IPO to identify colonies rapidly in agar (Van Vuurde, 1987b) with immunofluorescence colony staining. In immunofluorescence colony staining, extracts (e.g. seed leachate from beans) are mixed in different ratios (volume ratio of extract to agar e.g. 1:2 and 1:100) with the agar in petri dishes (for details see figure 1). Twenty-four to 48 hours after plating, colonies are formed in the agar, and the agar or part of the agar is dried under a warm air blower, followed by incubation with diluted FITC-conjugated antiserum (direct IF) for two hours-overnight. After several washing steps the agar can be checked at low magnification for fluorescing colonies. When the antiserum

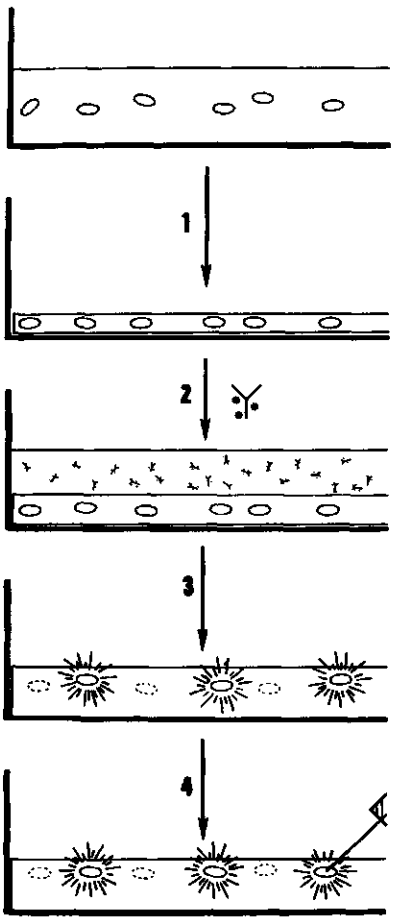

Figure 1. Principle of immunofluorescence colony staining in petri dishes or wells of tissue culture plates. Mix bacteria with the agar medium and incubate for 24-48 hours. 1. Dry the agar with a warm air blower (ca. 35 °C). 2. Add specific FITC-labelled antibodies and incubate overnight. 3. After incubation wash the plates 2 x 5 min with 0.01 M PBS, pH 7.6, and observe colonies with a UV microscope suited for IF at low ocular (4 or 6.3 x) and objective (4 or 6.3 x) magnification. 4. If isolation and further confirmation tests are needed, use needle or capillary for isolation of pathogen cells from the IF-positive colony. From Van Vuurde (1990a).

has been properly tested, colonies of the pathogen can be reliably identified in this way. The test can also be used to find cross-reacting colonies in research with antisera of unknown quality. In ordinary plating assays, in which usually 50-100 μl is plated on top of the medium in 9 cm petri dishes, a maximum of 300 colonies per plate is acceptable (ISO, 1978). Higher numbers per plate do not give reliable results. Immunofluorescence colony staining makes it possible to mix up to equal volumes of agar and seed extract (e.g. 5 ml of agar and 5 ml of seed extract), which results in a 100-1000 fold increase of

pinhead-size colonies growing out in the three-dimensional space of the medium. Colonies of the target bacterium can be recognised after staining with homologous antibodies, conjugated with a fluorescent dye. Bacteria inside the colonies survive the procedure and can still be isolated from positive colonies with a glass capillary. In practice, only a part of the agar in a petri dish is stained or the petri dish is replaced by e.g. the well of a 24 well tissue culture plate for efficiency and economy. When using these 24 well plates, each well is filled with ca. 400 μl agar and seed extract, mixed as mentioned before. Each well can contain a different sample or sample dilution. The optimum antiserum dilution is generally 1-2 dilution steps (2-fold dilutions) lower than the dilution suited for IF cell staining. Colony characteristics are less obvious when colonies are growing in rather than on the agar. Polyclonal antisera, e.g. produced against whole living bacteria in rabbits, are commonly used in immunofluorescence colony staining. Besides a normal four or 6.3 times objective, the materials needed for immunofluorescence colony staining, such as microscopes, filtersystems, buffers and conjugates are the same as for IF. The important aspects of procedures and materials, needed for IF, are discussed elsewhere (ISTA, 1988).

Good results were obtained for *Pseudomonas syringae* pv. *phaseolicola* (figure 2) and *Clavibacter michiganensis* subsp. *michiganensis* at RPvZ and IPO. Especially, for *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker in tomato (*Lycopersicon lycopersicum* (L.) Karsten en Farw.), this test may become very useful since the infection level of tomato seeds is often very low, whereas relatively large numbers of saprophytes are present.

Direct double diffusion

As for immunofluorescence colony staining, double diffusion directly done in agar may also help to confirm colonies more quickly (Contreras de Velasquez and Trujillo, 1984; Van Vuurde, 1987b). After 24-48 hours incubation of the dilution plates colonies on the agar are observed with a binocular at low magnification. Wells of 4 mm diameter are then made in the agar medium at a distance of ca. 10 mm from the colonies to be tested and antiserum is added to the wells in a relatively low dilution (e.g. 1:2 to 1:8).

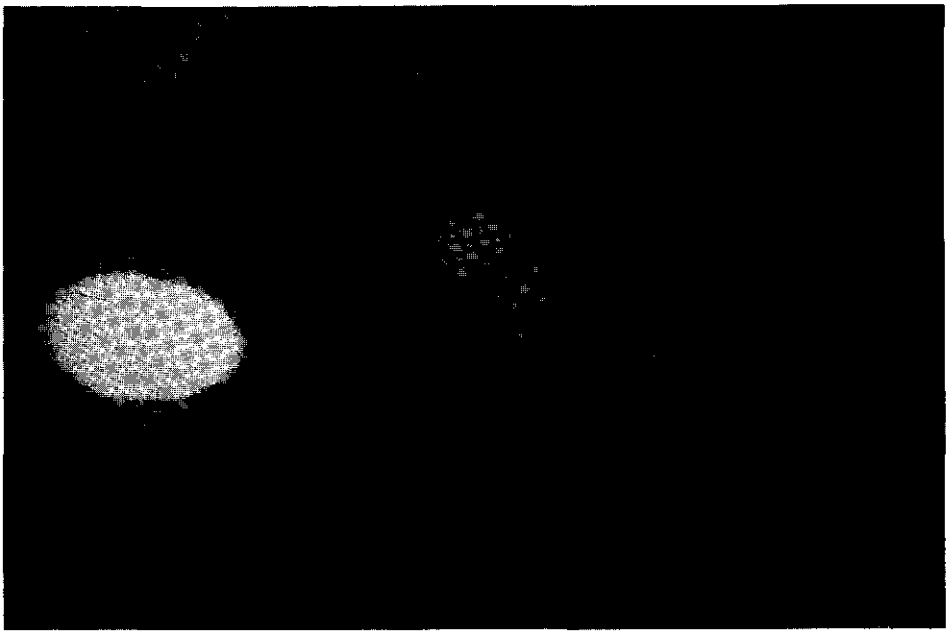

Figure 2. Immunofluorescence colony staining of *P.s.* pv. *phaseolicola* (P) among unstained saprophyte colonies (S). Before staining a soaking solution of an infected bean seed lot was mixed with King's medium B and incubated for 48 hours.

Antisera are diluted in 0.01 M PBS, pH 7.2. Dependent on the bacterial pathogen and the rate of colony growth on the medium, precipitation lines in general become visible within 24-48 hours. Since different soluble antigens from the target bacterium may continue to be formed for many days, plates are further incubated (eventually at 4 °C to stop colony growth) for a final inspection after one week. The amount of wells in a plate varies from 1-10, depending on the number and the place of the colonies on the agar. The antiserum per well amounts ca. 5 μl. This test is especially interesting for pathogens which are hard to recognise on the base of colony morphology and for samples with high saprophyte numbers, since precipitation lines are also formed in the direct presence of saprophyte colonies.

For direct double diffusion (and immunofluorescence colony staining), one has to study the application of this test for every individual pathogen, since antigens and antisera may vary considerably. Investigations are now concentrated on the application of this test in

the detection of *Pseudomonas syringae* pv. *phaseolicola* on King's medium B and on a semi-selective medium like MSP. The risk of false positive reactions with antiserum which is properly tested, is very low and can be further reduced by isolation of bacteria from serologically positive colonies, followed by other confirmation tests. Furthermore, the direct double diffusion and immunofluorescence colony staining method can be used to detect and isolate cross-reacting bacteria. These organisms can be employed to improve the quality of the antiserum (e.g. saturation of the antiserum according to Clausen (1981)) and for comparative studies to characterise typical antigenic determinants of the target bacterium.

Immunosorbent immunofluorescence and immuno-isolation tests

Antibodies can be used to selectively trap bacteria from plant material (Ruissen, Helderman, Schipper and Van Vuurde, 1987; Van Vuurde, 1987b; Van Vuurde en Van Henten, 1983; Van Vuurde et al., 1987). This principle is used in immunosorbent immunofluorescence and immuno-isolation. Immunosorbent immunofluorescence and immuno-isolation were presented and discussed by Van Vuurde and Van Henten (1983). Details of the procedure of immuno-isolation were evaluated by Stead, Chauveau, Janse, Ruissen, Van Vaerenbergh and Van Vuurde (1988). The principle and details of immuno-isolation are given in figure 3. Various materials such as glass rods treated with cellulose nitrate, petri dishes, microplates and polystyrene beads, can be used as the solid phase for coating with antibodies.

Immunosorbent immunofluorescence and immuno-isolation assays offer the possibility for selective trapping of the pathogen and/or cross-reacting cells from plant material, containing high numbers of saprophytic micro-organisms. In IF, much 'cleaner' slides are obtained. In immuno-isolation assays, media with a high plating efficiency for the target bacterium could be used, since target bacteria are selectively trapped (and concentrated) from a (seed) extract. Immuno-isolation tests are now also applied at IPO for isolation of cross-reacting micro-organisms which are used to evaluate the quality of polyclonal and monoclonal antisera.

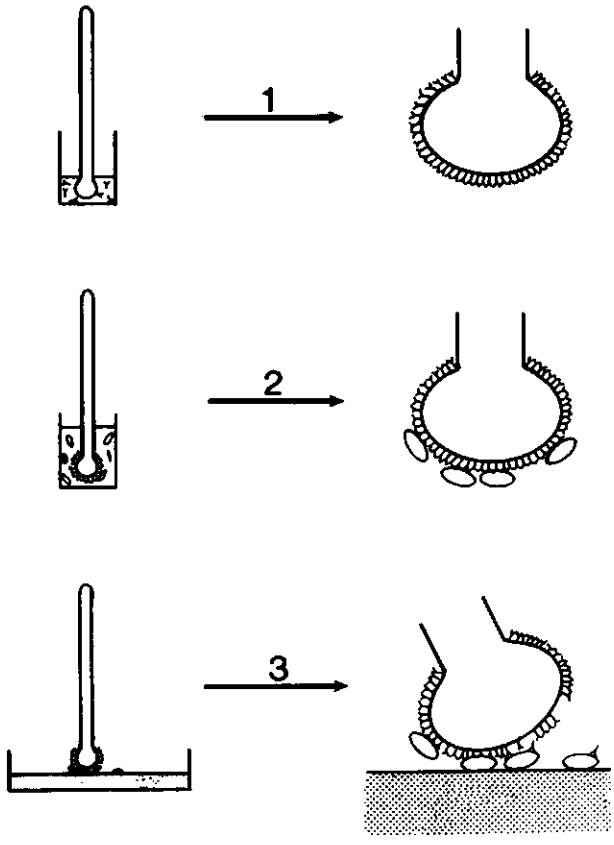

Figure 3. Principle of immuno-isolation with rods. 1. Coating of a polystyrene rod with antibodies against surface antigens of the target bacterium. The coating procedure is comparable to that of ELISA (Clark and Adams, 1977). Wash vigorously after coating. 2. Incubation of the antibody-coated rod in the sample to trap the target bacteria (e.g. one hour at room temperature). Wash very gently after incubation to remove non-bound organisms. 3. Mechanical disruption of the antibody-antigen linkage on the agar surface to prepare a colony density gradient of the trapped bacteria on one or more agar medium plates.
From Van Vuurde (1990b).

For *Clavibacter michiganensis* subsp. *michiganensis* and *Pseudomonas syringae* pv. *phaseolicola* a maximum of 5-6 fold increase, as percentage of colonies in a mixed suspension, was obtained when rods which were coated with the homologous antiserum were used to trap the pathogen selectively (Van Vuurde and Van Henten, 1983). Moreover, saprophyte to pathogen ratios (S/P) can change dramatically. Ruissen et al. (1987) obtained a change in S/P ratio from 50:20 to 2:50 when a Sepharose 6 MB column, coated with anti-*Clavibacter michiganensis* subs. *michiganensis* serum, was used.

Discussion and conclusions

For all serological tests, more work is needed to improve and to obtain more standardisation in methods for producing and testing antisera. Isolation of specific antigens and the production of monoclonal antibodies may be very useful with respect to specificity of the antisera (Schaad, 1982). More standardisation is also needed in working procedures of the serological tests and more basis research is needed to find suitable antibodies for each individual serological test. To improve the sensitivity of serological tests one might think of enrichment procedures, needed to accumulate the target bacterium or metabolites of the target bacterium (Beguin, Gugerli and Cazelles, 1984; Trujillo and Saettler, 1979). For a serological test such as ELISA specific improvements of the sensitivity could be found in e.g. enzyme amplification, in which the enzyme substrate reaction differs from the conventional double antibody sandwich ELISA (Cooper and Edwards, 1986; Torrance, 1986).

Since serological tests have many advantages over other tests and since serological tests can be used in many ways and in combination with other tests in the identification or detection of seed-borne bacteria, we believe further research is justified. In spite of the drawbacks mentioned before, serological tests are potentially most suited for screening and identification in seed bacteriology as long as antisera are tested properly and used accordingly.

The immunofluorescence colony staining, direct double diffusion and immuno-isolation tests have good prospects for routine application in seed bacteriology. However, more

research is needed to find optimal formats and materials for routine tests (Van Vuurde, 1987b).

References

Akerman, A., Zutra, D., Volcani, Z. and Henis, Y., 1973. Application of an immunofluorescent technique for detecting *Corynebacterium michiganense* and estimating its extent in tomato seed lots. Phytoparasitica 1: 128.

Alvarez, A.M. and Lou, K., 1985. Rapid identification of *Xanthomonas campestris* pv. *campestris* by ELISA. Plant Disease 69: 1082-1089.

Alvarez, A.M., Benedict, A.A. and Mizumoto, C.Y., 1985. Identification of xanthomonads and grouping of strains of *Xanthomonas campestris* pv. *campestris* with monoclonal antibodies. Phytopathology 75: 722-728.

Barzic, M. and Trigalet, A., 1982. Détection de *Pseudomonas phaseolicola* (Burkh.). Dowson par la technique ELISA. Agronomie 2: 389-398.

Beguin, N., Gugerli, P. and Cazelles, O., 1984. Detection d'infections latentes de jambe noire (*Erwinia carotovora* var. *atroseptica*) dans les tubercules de pomme de terre par le dosage immuno-enzymatique ELISA. Revue suisse Agric. 16: 321-324.

Clark, M.F. and Adams, A.N., 1977. Characteristics of the microplate method of enzyme-linked immuno-sorbent assay for the detection of plant viruses. Journal of General Virology 34: 475-483.

Clausen, J., 1981. Immunochemical techniques for the identification and estimation of macromolecules, pp. 225. Elsevier, Amsterdam.

Coleno, A., 1968. Utilisation de technique d'immunofluorescence pour le dépistage de *Pseudomonas phaseolicola* (Burkh.). Dowson dans les lots de semences de haricots contaminés. Comptes Rendu Academie Agriculture France 54: 1016-1024.

Coleno, A., Trigalet, A. and Digat, B., 1976. Detection des lots de semences contaminés par une bacterie phytopathogene. Annales de Phytopathologie 8: 355-364.

Contreras de Velasquez, N. and Trujillo, G.E., 1984. Evaluacion de *Xanthomonas campestris* pv. *phaseoli* (Smith) Dye. en lotes de semilla de caraota (*Phaseolus vulgaris*

L.) mediante la tecnica combinada del medio semi-selectivo en immunodiffusion en agar [Evaluation of *Xanthomonas campestris* pv. *phaseoli* (Smith) Dye. in bean (*Phaseolus vulgaris* L.) seed lots by the combined technique of a semi-selective medium and immunodiffusion in agar]. Agronomia Tropical 34: 59-67.

Cooper, J.I. and Edwards, M.L., 1986. Variations and limitations of enzyme-amplified immunoassays. In: Developments and applications in virus testing, (eds. R.A.C. Jones and L. Torrance), pp. 139-154. AAB Office, Wellesbourne, United Kingdom.

De Boer, S.H. and Mc Naughton, M.E., 1987. Monoclonal antibodies to the lipopolysaccharide of *Erwinia carotovora* subsp. *atroseptica* serogroup I. Phytopathology 77: 828-832.

Elango, F. and Lozano, J.C., 1980. Transmission of *Xanthomonas manihoitis* in seed of cassava (*Manihoit esculenta*). Plant Disease 64: 784-786.

Erwin, D.C. and Khan, R.A., 1987. Detection of *Clavibacter michiganense* pv. *insidiosum* in alfalfa seed by isolation and ELISA. Phytopathology 77: 1706 (Abstr.).

Guthrie, J.W., 1986. The serological relationship of races of *Pseudomonas phaseolicola*. Phytopathology 58: 716-717.

Guthrie, J.W., Huber, D.M. and Fenwick, H.S., 1965. Serological detection of halo blight. Plant Disease Reporter 4: 297-299.

ISO, 1978. Microbiology - General guidance for enumeration of micro-organisms - Colony count technique at 30 °C. International Organization for Standardisation, ref. no. ISO 4833-1978 (E), UDC 663.1.

ISTA, 1981. Report of 17th International Seminar on Seed Pathology, 1981. Zürich. International Seed Testing Association, Zürich, Switzerland.

ISTA, 1982. Report of 1st International Workshop on Seed Bacteriology, 1982. Angers. International Seed Testing Association, Zürich, Switzerland.

ISTA, 1984. Report of 18th International Seminar on Seed Pathology, 1984. Washington State University, Puyallup. International Seed Testing Association, Zürich, Switzerland.

ISTA, 1988. Report of 1st International Serology Workshop, 1987. Wageningen. International Seed Testing Association, Zürich, Switzerland.

Lamka, G.L., McGee, D.C., Hill, J.H. and Braun, E.J., 1987. Development of an

immunosorbent assay for seed borne *Erwinia stewartii*. Phytopathology 77: 1706 (Abstr.).

Malin, E.M., Roth, D.A. and Belden, E.L., 1983. Indirect immunofluorescent staining for detection and identification of *Xanthomonas campestris* pv. *phaseoli* in naturally infected bean seed. Plant Disease 67: 645-647.

Minsavage, G.V. and Schaad, N.W., 1983. Characterization of membrane proteins of *Xanthomonas campestris* pv. *campestris*. Phytopathology 73: 747-755.

Ruissen, M.A., Helderman, C.A.J., Schipper, J. and Van Vuurde, J.W.L., 1987. Selective isolation and concentration of phytopathogenic bacteria on immunoaffinity columns. In: Proceedings of the 6th International Conference on Plant Pathogenic Bacteria (eds. E.L. Civerolo, A. Collmer, R.E. Davis and A.G. Gillaspie), pp. 882. Martinus Nijhoff Publishers. Dordrecht/Boston/Lancaster.

Schaad, N.W., 1978. Use of direct and indirect immunofluorescence tests for identification of *Xanthomonas campestris*. Phytopathology 68: 249-252.

Schaad, N.W., 1979. Serological identification of plant pathogenic bacteria. Annual Review of Phytopathology 17: 123-147.

Schaad, N.W., 1982. Detection of seedborne bacterial plant pathogens. Plant Disease 66: 885-890.

Sheppard, J.W., 1983. Detection of seed-borne bacterial blights of bean. Seed Science and Technology 11: 561-567.

Sheppard, J.W., Wright, P.F. and DeSavigny, D.H., 1986. Methods for the evaluation of EIA tests for use in the detection of seed-borne diseases. Seed Science and Technology 14: 49-59.

Stead, D.E., Chauveau, J.F., Janse, J.D., Ruissen, M.A., Van Vaerenbergh, J. and Van Vuurde, J.W.L., 1988. Immuno-isolation techniques for the detection and isolation of plant-pathogenic bacteria. In: J.M. Grange, A. Fox and N.L. Morgen (eds.), Immunological techniques in microbiology, pp. 189-193. Society for Applied Bacteriology.

Tanii, A., Takakuwa, M., Baba, T. and Takita, T., 1976. Studies on halo blight of beans (*Phaseolus vulgaris*) caused by *Pseudomonas phaseolicola* of (Burkholder) Dawson.

Miscellaneous publication of Hokkaido prefectural Tokachi agricultural experiment station, no. 6.

Taylor, J.D., 1970. Bacteriophage and serological methods for identification of *Pseudomonas phaseolicola* (Burkh.) Dowson. Annals of Applied Biology 66: 387-395.

Thaveechai, N. and Schaad, N.W., 1986a. Serological and electrophoretic analysis of a membrane protein extract of *Xanthomonas campestris* pv. *campestris* from Thailand. Phytopathology 76: 139-147.

Thaveechai, N. and Schaad, N.W., 1986b. Immunochemical characterization of a subspecies-specific antigenic determinant of a membrane protein extract of *Xanthomonas campestris* pv. *campestris*. Phytopathology 76: 148-153.

Torrance, L., 1986. Use of enzyme amplification in an ELISA to increase sensitivity of detection of barley yellow dwarf virus in oats and in individual vector aphids. Journal of Virological Methods 15: 131-138.

Trigalet, A. and Bidaud, P., 1978. Some aspects of epidemiology of bean halo blight. Proceedings of the Fourth International Conference on Plant Pathogenic Bacteria, Angers, pp. 895-902.

Trigalet, A. and Rat, B., 1976. Immunofluorescence as a tool for detecting the internally borne bacterial diseases: *Corynebacterium michiganense* (E.F. Smith) Jensen and *Pseudomonas phaseolicola* (Burkholder) Dowson. Report of 15th International Workshop on Seed Pathology, Paris. International Seed Testing Association, Zürich, Switzerland.

Trigalet, A., Samson, R. and Coleno, A., 1978. Problems related to the use of serology in phytobacteriology. Proceedings of the Fourth International Conference on Plant Pathogenic Bacteria, Angers, pp. 271-288.

Trujillo, G.E. and Saettler, A.W., 1979. A combined semi-selective medium and serology test for the detection of *Xanthomonas* blight bacteria in bean seed. Journal of Seed Technology 4: 35-41.

Van Vuurde, J.W.L., 1987a. Detecting seedborne bacteria by immunofluorescence. In: Proceedings of the 6th International Conference on Plant Pathogenic Bacteria (eds. E.L. Civerolo, A. Collmer, R.E. Davis and A.G. Gillaspie), pp. 779-808. Martinus Nijhoff

Publishers. Dordrecht/Boston/Lancaster.

Van Vuurde, J.W.L., 1987b. New approach in detecting phytopathogenic bacteria by combined immunoisolation and immunoidentification assays. Bulletin OEPP/EPPO 17: 139-148.

Van Vuurde, J.W.L., 1990a. Immunofluorescence colony staining. In: R. Hampton, E. Ball and S. de Boer (eds.), Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens, pp. 299-305. APS Press, St. Paul, Minnesota, USA.

Van Vuurde, J.W.L., 1990b. Immuno-isolation. In: Z. Klement, K. Rudolph and D.C. Sands (eds.), Methods in Phytobacteriology, pp. 169-172. Akadémiai Kiadó, Budapest, Hungary.

Van Vuurde, J.W.L. and Van Henten, C., 1983. Immunosorbent immunofluorescence microscopy (ISIF) and immunosorbent dilution-plating (ISDP): New methods for the detection of plant pathogenic bacteria. Seed Science and Technology 11: 523-533.

Van Vuurde, J.W.L., Ruissen, M.A. van Vruggink, H., 1987. Principles and prospects of new serological techniques including immunosorbent immunofluorescence, immunoaffinity isolation and immunosorbent enrichment for sensitive detection of phytopathogenic bacteria. In: Proceedings of the 6th International Conference on Plant Pathogenic Bacteria (eds. E.L. Civerolo, A. Collmer, R.E. Davis and A.G. Gillaspie), pp. 835-842. Martinus Nijhoff Publishers. Dordrecht/Boston/Lancaster.

Van Vuurde, J.W.L., Van den Bovenkamp, G.W. and Birnbaum, Y., 1983. Immunofluorescence microscopy and enzyme-linked immunosorbent assay as potential routine tests for the detection of *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* in bean seed. Seed Science and Technology 11: 547-559.

Weaver, W.M. and Guthrie, J.W., 1978. Enzyme linked immunospecific assay: Application to the detection of seed-borne bacteria. Phytopathology News 12: 156 (Abstr.).

Chapter 8. General Discussion

General Discussion

The use of healthy crucifer seed is an important way to control the black rot disease, caused by *Xanthomonas campestris* pv. *campestris*. Therefore, methods for detecting *X. c.* pv. *campestris* in crucifer seed lots are needed.

The detection of *X. c.* pv. *campestris* by plating assays and immunofluorescence microscopy (IF) was studied in detail. Some important aspects of an isolation assay for *X. c.* pv. *campestris* were discussed (chapter 3). The recently developed CS20ABN medium (Chang et al., 1991) may be advantageous for more reliable detection of this pathogen. Recent investigations show that the merit of this medium may, however, also depend on the seed lot tested and the extraction method used. Recently, ten seed lots tested in earlier experiments (chapter 3 and 5) were indexed for presence of *X. c.* pv. *campestris* using 5 min and 2.5 h shaking of seed lots. Seed washings were plated on NSCAA and CS20ABN (A.A.J.M. Franken, unpublished). Totally, four seed lots were found positive on NSCAA and three on CS20ABN. NSCAA and CS20ABN generally did not differ with respect to number of colony-forming units of saprophytes and *X. c.* pv. *campestris*. However, *X. c.* pv. *campestris* colonies were generally larger on CS20ABN than on NSCAA. In other tests with different seed lots, colonies from the CS20ABN medium were easier to recognize and less saprophytes were found on the CS20ABN than on the NSCAA medium (J. van Bilsen, Bejo Seeds B.V., Warmenhuizen, the Netherlands, personal communication), which is in agreement with the report given by Chang et al. (1991). These contradictory results may be explained by the fact that in the study mentioned above, seed lots were stored for nearly 6 years. During storage bacteria may be damaged or weakened and growth of these bacteria may be inhibited when directly plated onto selective media such as CS20ABN. When plating on NSCAA, reported to be less selective than CS20ABN (Chang et al., 1991) but most useful for isolating *X. c.* pv. *campestris* from seeds (Schaad and Stall, 1988), bacteria may recover and grow more easily. Thus, in some instances also the CS20ABN medium may not detect *X. c.* pv. *campestris*. In spite of the use of selective media, saprophytes or other

factors may still interfere with plating assays, because substances, toxic for *X. c.* pv. *campestris*, may be released in the extraction fluid. To avoid false-negative reactions in plating assays it is advisable to use more than one medium (Randhawa and Schaad, 1984), even when the CS20ABN medium is used. To solve the problems relating to the detection of *X. c.* pv. *campestris* by plating assays in the long run, more information is needed on the significance of the interference by saprophytes and antibacterial substances (Schaad and Donaldson, 1980, 1981). Besides bacterial antagonists also fungal antagonists and chemical substances, produced by the seed (Malekzadeh, 1966) should be taken into consideration.

Chapter 5 and 6 showed that when using two extraction methods (5 min and 2.5 h shaking at room temperature) for each seed lot the risk of false-negative results in IF could be minimized but not completely excluded. Recently, some seed lots were found completely negative in IF with monoclonal antibody MCA 20H6, but strongly positive with MCA 2F4, MCA 18G12 and PCA 94 (A.A.J.M. Franken, unpublished; J. van Bilsen, Bejo Seeds B.V., Warmenhuizen, The Netherlands, personal communication). Of the isolates obtained from one of these seed lots, none of the *X. c.* pv. *campestris* isolates reacted well with MCA 20H6, whereas nearly all isolates reacted with MCA 2F4, MCA 18G12, MCA 10C5 and PCA 94 in IF (n=32). In a dot-blot immunoassay (DBI), more than 72% of all isolates reacted with all the antibodies tested. These results indicate that the homologous antigen (the lipopolysaccharide) is not or variably exposed in some seed lots and on plating media, when IF is used for detection and identification of the black rot pathogen. However, when using DBI for identification of pure cultures, the antigen may be detected since the epitopes are less variably exposed, as was also discussed in chapter 4. Although in chapter 5 it was reported that using a mixture of antibodies does not result in more fluorescent cells in IF, for some seed lots a mixture of monoclonal antibodies proved to be useful in avoiding false-negative results. Applying a mixture of MCA 20H6, 2F4 and 18G12 for the seed lot mentioned above would have led to a correct detection of fluorescent cells in seed extracts and a correct identification of 100% of the isolates in IF and in DBI (A.A.J.M. Franken, unpublished).

When using IF and dilution-plating for direct detection of *X. c.* pv. *campestris* in seed

washings, problems may arise when IF and dilution-plating give results that do not correspond (chapter 6). Generally, seed lots positive in dilution-plating will very likely show disease in the field under optimum conditions, as was shown by Schaad (1983) for *X. c.* pv. *campestris* and Van Vuurde et al. (1991) for *Pseudomonas syringae* pv. *phaseolicola*. However, dilution-plating may not detect all infected seed lots (Schaad, 1983; Van Vuurde et al., 1991). On the basis of data provided by Schaad (1983) and Van Vuurde et al. (1991), dilution-plating would not detect 8-20% of the infected seed lots. On the same basis, it was shown that 100% of the IF-negative seed lots and 30-50% of the IF-positive seed lots did not give disease in the field (Schaad, 1983; Van Vuurde et al., 1991). The prevalence of the disease ('the amount of disease present in a specified population at a given point of time'; Sheppard et al., 1986) has not been considered in these data.

If the diagnostic sensitivity ('a measure of the ability of a positive test to correctly identify a seed lot with a specified disease when the disease status of the seed lot is known'; Sheppard et al., 1986) and the diagnostic specificity ('a measure of the ability of a negative test to correctly identify healthy seed lots, i.e. those free from the specified disease when the disease status is known'; Sheppard et al., 1986) are known, the predictive value of a positive and negative result can be calculated, given a specified prevalence of the disease. The predictive value indicates the probability that given a negative or positive result, the sample is truly healthy or infected (positive), respectively.

The prevalence of the black rot disease in the experiments reported in this thesis was estimated. The prevalence in experiment 1 (chapter 6, table 1 and 2) was estimated to be c. 50%, in experiment 2 and 3 c. 20% (chapter 6, table 1 and 2). The diagnostic sensitivity of IF and dilution-plating, as extrapolated from data given by Schaad (1983) and Van Vuurde et al. (1991), was estimated to be minimally 99.9% and 80-90%, respectively. The diagnostic specificity for IF and dilution-plating is estimated to be minimally 50-70% and 99.9%, respectively.

With these figures the predictive value of a negative result and positive result can be calculated (table 1). Table 1 confirms the idea that an analytical sensitive technique such as IF is highly suited for screening purposes, since the risk of false-negative results is

very low. The major implications of table 1 are that in experiment 1 (prevalence of c. 50%, chapter 6) 23.1-33.3% of the IF-positive results (which were 78-85% of all samples tested) would have been false-positive, as compared to field incidence of black rot. In experiment 2 and 3 (prevalence of c. 20%) 54.6-67.0% of the IF-positive results (which were 39-59% of all samples tested) would have been false-positive, as compared to field incidence of black rot. Other major implications are that in experiment 1 (chapter 6), 9.1%-16.7% of the dilution-plating negative samples (54-62% of all samples tested were negative in dilution-plating) are false-negative, as compared to field incidence of black rot. In experiment 2 and 3 the percentage would be 3.4-4.8% (80-85% of all samples tested were negative in dilution-plating), as compared to field incidence of black rot. Table 1 also shows that, when the prevalence of a disease is relatively low, the predictive value of a positive result is relatively low, and for a negative result is relatively high. When the prevalence of the disease is high the contrary is valid. IF gives an exceptionally good prediction for 'health' (high predictive value of a negative result). Dilution-plating gives an exceptionally good prediction for 'disease' (a high predictive value of a positive result). This confirms results given by Van Vuurde et al. (1991).

It should be noted that all data are calculated for a predictive value of disease or health, and not for a comparison with a test approaching 100% diagnostic specificity and sensitivity, which should be preferred (Sheppard et al., 1986). This implies that in reality at least a part of the false-positives may have been 'real positives'. In practice seed lots may be found infected but may not give any infected plants because the rate of seed-transmission (the percentage of infected plants arising from infected seeds) is not 100% and environmental conditions are suboptimal. The rate of seed-transmission for seed lots infected with *X. c.* pv. *campestris* is 14.25-65.0%, as calculated from data provided by Schaad (1983). Moreover, low numbers of fluorescent cells in IF indicate the presence of the pathogen at a very low level. This level is often not high enough to yield infected plants.

In this thesis some critical factors of the detection of *X. c.* pv. *campestris* in crucifer seeds by IF and dilution-plating were evaluated. To minimize the risk of false-negative results, leading to an unacceptable disease development in the field, it is advisable to use

Table 1. Effect of prevalence on the predictive value of immunofluorescence microscopy (IF) and dilution-plating for detecting *Xanthomonas campestris* pv. *campestris* in crucifer seeds.

Prevalence of the black rot disease (%)	Predictive value[1] of a positive (field) test (%)		Predictive value[1] of a negative (field) test (%)	
	IF	dilution-plating	IF	dilution-plating
1	1.98-3.25	89.0-90.1	100-100	99.8-99.9
15	26.1-37.0	99.3-99.4	100-100	96.6-98.3
20	33.0-45.4	99.5-99.6	100-100	95.2-97.6
30	46.1-58.8	99.7-99.7	99.9-99.9	92.1-95.9
40	57.1-68.9	99.8-99.8	99.9-99.9	88.2-93.7
50	66.7-76.9	99.9-99.9	99.8-99.9	83.3-90.9

[1]Predictive values were calculated as described by Sheppard et al. (1986) with the values found by Schaad (1983) and Van Vuurde et al. (1991): diagnostic sensitivity of IF and dilution-plating is 99.9% and 80-90%, respectively, and diagnostic specificity of IF and dilution-plating is 50-70% and 99.9%, respectively.

at least two plating media (e.g. NSCAA and CS20ABN) in dilution-plating and two extraction methods (5 min and 2.5 h shaking). For IF, it is advisable to test with different monoclonal antibodies separately, a mixture of monoclonal antibodies (e.g. MCA 20H6, MCA 2F4 and MCA 18G12), or both a monoclonal antibody (MCA 20H6 or 2F4) and a high quality polyclonal antiserum (PCA 94).

When a prediction of a negative result ('health') is needed, IF is more suited. However, there is a relatively larger risk for false-positive results (as compared to field incidence of black rot) especially at low prevalence of the disease. When a prediction of a positive result ('disease') is needed, dilution-plating is more suited. However, there is a relatively large risk for false-negative reactions, especially at high prevalence of the disease.

To use both the high diagnostic sensitivity of IF and high diagnostic specificity, other ways to combine both techniques should be investigated. For this purpose, e.g. the development of an immunofluorescence colony-staining or an immuno-isolation technique may be considered (chapter 7).

References

Alvarez, A.M., Benedict, A.A. & Mizumoto, C.Y., 1985. Identification of xanthomonads and grouping of strains of *Xanthomonas campestris* pv. *campestris* with monoclonal antibodies. Phytopathology 75: 722-728.

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

Malekzadeh, F., 1966. An antibacterial substance from cauliflower seeds. Phytopathology 56: 497-501.

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

Schaad, N.W., 1983. Correlation of laboratory assays for seedborne bacteria with disease development. Seed Science and Technology 11: 877-883.

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

Schaad, N.W. & Donaldson, R.C., 1981. Bacteria of crucifer seeds antagonistic to *Xanthomonas campestris*. Phytopathology 71: 902.

Schaad, N.W. & Stall, R.E., 1988. D. Xanthomonas. In: Laboratory guide for identification of plant pathogenic bacteria (ed. N.W. Schaad), p. 81-94.

Sheppard, J.W., Wright, P.F. & DeSavigny, D.H., 1986. Methods for the evaluation of EIA tests for use in the detection of seed-borne diseases. Seed Science and Technology 14: 49-59.

Van Vuurde, J.W.L., Franken, A.A.J.M., Birnbaum, Y. & Jochems, G. (1991). Characteristics of immunofluorescence microscopy and of dilution-plating to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed lots and for risk assessment of field incidence of halo blight. Netherlands Journal of Plant Pathology 97: 233-244.

Summary

Black rot is one of the most threatening diseases of crucifers. The causal agent of this disease is the bacterium *Xanthomonas campestris* pv. *campestris*. The bacterium attacks all cultivated brassicas, radishes and numerous weeds, and is able to survive on plant debris in the soil. The primary source of inoculum is often infected seed. The most important ways to control black rot are the use of resistant cultivars and the use of 'healthy' seed. To obtain healthy seed, chemical or physical seed treatments may be used. These seed treatments may, however, seriously damage the seed quality (germination), may cause phytotoxicity or may not sufficiently eliminate the black rot pathogen from the seed (chapter 2). To check the seed health, sensitive and specific methods are needed to detect *X. c.* pv. *campestris* in the seed. Several methods for detecting *X. c.* pv. *campestris* are summarized in chapter 2. The most commonly used assays are plating assays, in which seed washings are plated onto isolation media, such as BSCAA (basal starch cycloheximide agar with nitrofurantoin and vancomycin), CS20ABN (a starch medium with bacitracin, neomycin and cycloheximide), FS (a medium with starch trimethoprim, cephalexin, cycloheximide, methyl green), NSCA (nutrient starch cycloheximide agar), NSCAA (NSCA with nitrofurantoin and vancomycin), and SMA-medium (starch-methionine agar with cephalexin and nitrofurantoin). Serological assays such as enzyme immuno-assays, Ouchterlony double diffusion, agglutination and immunofluorescence microscopy (IF) can be used for identification of pure cultures of *X. c.* pv. *campestris*. Cross-reactions with other pathovars of *X. campestris* have, however, been reported. So far, IF using polyclonal antisera was the only serological technique employed for detecting *X. c.* pv. *campestris* in seed washings.

The aim of this study was to analyse important characteristics of IF and plating assays, and to improve their use for identification and detection of *X. c.* pv. *campestris* in crucifer seeds. Methods for the detection and identification of *X. c.* pv. *campestris* are reviewed in chapter 2. In chapter 3 some aspects of plating assays for isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds are discussed. Little

differences were found between results obtained with NSCA, NSCAA and FS medium. It was, however, noted that the performance of the media often depends on the seed lot and extraction method used. Therefore, probably other methods such as immunofluorescence microscopy (IF) are needed to confirm presence of *X. c.* pv. *campestris* with higher certainty. With the extraction methods 2.5 h shaking and 1.5 h soaking, more colony-forming units were recovered from some seed lots than with the standard 5 min shaking of seed lots. However, prolonged extraction did not result in finding more seed lots infected. However, the use of two methods for extracting *X. c.* pv. *campestris* from crucifer seed, will enhance the chance of isolating the pathogen from the seed (chapter 3 and 6).

In chapter 4 the specificity of polyclonal antisera and monoclonal antibodies for identification of *X. c.* pv. *campestris* is discussed. Polyclonal antisera reacted in IF with all strains of *X. c.* pv. *campestris* and other xanthomonads (e.g. *X. c.* pv. *vesicatoria* and *amoraciae*) at low dilution (1:100). Non-xanthomonads also reacted with 2 out of 3 polyclonal antisera at this dilution. At higher dilutions (1:900), however, most cross-reactions with non-xanthomonads disappeared as well as reactions with some strains of *X. c.* pv. *campestris* and other pathovars. Six monoclonal antibodies (MCA 17C12, MCA 16B5, MCA 20H6, MCA 2F4, MCA 18G12, MCA 10C5), produced against *X. c.* pv. *campestris* were tested in immunoblotting (IB), an enzyme immunoassay (EIA), dot-blot immunoassay (DBI) and IF. The monoclonal antibodies reacted with the lipopolysaccharide (MCA 20H6, 2F4, 18G12, and 10C5) or membrane proteins (MCA 17C12 and 16B5) of *X. c.* pv. *campestris* in IB. Two monoclonal antibodies (MCA 17C12 and 16B5) reacted with all xanthomonads tested in DBI, but not in IF and EIA. The other monoclonal antibodies (MCA 20H6, 2F4, 18G12, and 10C5) did not react with all strains of *X. c.* pv. *campestris* and did react with some other xanthomonads, such as *X. c.* pv. *vesicatoria* and *amoraciae*, in IF, EIA and DBI. It was concluded that some polyclonal antisera and monoclonal antibodies may be used for identification of (a group of) strains of *X. c.* pv. *campestris*. The question, whether monoclonal antibodies, as compared to a polyclonal antiserum, may give rise to false-negative or false-positive results when testing seed lots, is dealt with in chapter 5. IF with one polyclonal antiserum

and monoclonal antibodies was used for direct detection of *X. c.* pv. *campestris* in seed washings and identification of colonies. For direct detection of *X. c.* pv. *campestris* in seed washings, it was found that IF-results (cell counts) depend in part on the seed lot tested, the extraction method and antibodies used. This confirmed the interactions found in plating assays. The polyclonal antiserum tested (PCA 94) did not always result in detection of more fluorescent cells in IF. On the contrary, for some seed lots and extraction methods the opposite was found; monoclonal antibodies sometimes detected more fluorescent cells than polyclonal antisera. When using two extraction methods (5 min and 2.5 h shaking at room temperature) for one seed lot the risk of false-negative results in IF could be minimized (chapter 5 and 6). When monoclonal antibodies were used for identification of colonies, it was found that some *X. c.* pv. *campestris* colonies did not react with antibodies MCA 16B5, MCA 17C12, and to a lesser extent with MCA 10C5, MCA 2F4, and MCA 18G12. Saprophytic isolates of one seed lot cross-reacted with MCA 17C12 and to a lesser extent with MCA 2F4, 18G12 and PCA 94. On the basis of this study MCA 20H6 was considered to be the most suited antibody for detection and identification of *X. c.* pv. *campestris*.

Chapter 6 shows that the correlation between IF and dilution-plating was similar for one monoclonal antibody (MCA 20H6) and one polyclonal antiserum tested (PCA 94). With increasing cell numbers in IF the chance of isolating *X. c.* pv. *campestris* also increased. With IF generally much more seed lots were found positive than with dilution-plating. It was shown that the correlation between IF and dilution-plating depended in part on the volume of seed extract examined. When examining relatively large volumes (e.g. 50 μl), the sensitivity of IF will be enhanced and the risk of false-negative reactions in IF as compared to dilution-plating will be decreased. On the other hand the correlation with dilution-plating decreased. It was concluded that IF generally gives a good prediction of 'health' of a seed lot and that dilution-plating generally gives a good prediction of 'disease'. Chapter 7 summarizes the problems with serological techniques for seed-borne bacteria and gives possible solutions to be worked out in future.

To minimize the risk of false-negative results, it is advisable to use at least two plating media (e.g. NSCAA and CS20ABN) in dilution-plating and two extraction methods (5

min and 2.5 h shaking at room temperature) in both IF and dilution-plating. For IF, it is advisable to screen either with different monoclonal antibodies separately, with a mixture of monoclonal antibodies (e.g. MCA 20H6, MCA 2F4 and MCA 18G12), or both a monoclonal antibody (MCA 20H6 or 2F4) and a high quality polyclonal antiserum (PCA 94).

More research is needed, however, to assess the significance of interference by saprophytes and antibiotic substances, released by the seed or saprophytes, with the detection assay and the survival of the pathogen in the seed.

Samenvatting

Zwartnervigheid is één van de meest bedreigende ziekten van kruisbloemigen. Deze ziekte wordt veroorzaakt door de bacterie *Xanthomonas campestris* pv. *campestris*. Deze bacterie kan alle gecultiveerde kool- en radijsachtigen, alsmede kruisbloemige onkruiden, aantasten en is in staat om in de grond op resten van planten te overleven. De primaire bron van infectie is vaak besmet zaad. De belangrijkste manier om zwartnervigheid te voorkomen en te beheersen is het gebruik van resistente cultivars en gezond zaad. Teneinde het zaad "gezond" te maken, kan overwogen worden om het zaad chemisch of fysisch te behandelen. Deze behandelingen kunnen echter de zaadkwaliteit (kiemkracht) aantasten en fytotoxiciteit veroorzaken. Daarnaast zijn de behandelingen soms niet in staat om alle cellen van de ziekteverwekker in het zaad te doden.

Om de gezondheid van het zaad te bepalen zijn gevoelige en specifieke methoden nodig die *X. c.* pv. *campestris* in zaadextracten kunnen aantonen. Verschillende methoden om *X. c.* pv. *campestris* aan te tonen worden in hoofdstuk 2 samengevat. De meest gebruikte methoden zijn uitplaattechnieken, waarbij week- of schudvloeistoffen afkomstig van zaden uitgeplaat worden op media zoals BSCAA (een basismedium met zetmeel, cycloheximide, nitrofurantoïne en vancomycine), CS20ABN (een medium met bacitracine, neomycine en cycloheximide), FS (een medium met zetmeel, trimethoprim, cephalexine, cycloheximide en methylgroen), NSCA (nutriënt agar met zetmeel en cycloheximide), NSCAA (NSCA met nitrofurantoïne en vancomycine) en SMA-medium (zetmeel-methionine agar met cephalexine en nitrofurantoïne). Serologische technieken zoals "enzyme immunoassays", Ouchterlony dubbele diffusie, agglutinatie en immunofluorescentie microscopie (IF) kunnen gebruikt worden voor identificatie van *X. c.* pv. *campestris* kolonies. Kruisreacties met andere pathovars van *Xanthomonas campestris* kunnen echter ook optreden. Van deze serologische technieken werd tot nu toe alleen IF m.b.v. polyklonale antisera gebruikt voor de detectie van *X. c.* pv. *campestris* in zaadextracten.

Het doel van dit onderzoek was om een aantal belangrijke eigenschappen van IF en uitplaatmethoden te analyseren en het gebruik van deze methoden voor de identificatie en

detectie van *X. c.* pv. *campestris* in zaden van kruisbloemigen te verbeteren. Hoofdstuk 3 behandelt een aantal belangrijke aspecten van een uitplaatmethode voor de isolatie van *X. c.* pv. *campestris* uit zaden van kruisbloemigen. Er werden slechts geringe verschillen gevonden tussen de resultaten behaald met het NSCA, NSCAA en FS medium. Wél werd opgemerkt dat de werkzaamheid van de media vaak afhangt van de onderzochte zaadpartij en de gebruikte extractiemethode. Dit betekent dat waarschijnlijk andere methoden, zoals IF, nodig zijn om de aanwezigheid van X. c. pv. *campestris* met grotere zekerheid te bevestigen. Met de gebruikte extractiemethoden, 2,5 uur schudden en 1,5 uur weken, werden voor sommige zaadmonsters meer kolonievormende eenheden gevonden dan met de standaard gebruikte methode van 5 minuten schudden. Echter deze verlengde extractie leidde niet tot het vinden van meer positieve (besmette) zaadmonsters. Wanneer echter twee extractiemethoden in combinatie gebruikt worden, b.v. 5 minuten en 2,5 uur schudden bij kamertemperatuur, wordt de kans op detectie van *X. c.* pv. *campestris* m.b.v. de uitplaatmethode groter.

In hoofdstuk 4 wordt de specificiteit van polyklonale antisera (PKA) en monoklonale antilichamen (MKA) voor de identificatie van *X. c.* pv. *campestris* besproken. Bij lage verdunningen (1:100) reageerden de polyklonale antisera in IF met alle stammen van *X. c.* pv. *campestris* en andere xanthomonaden (b.v. *X. c.* pv. *vesicatoria* en *amoraciae*). Bij deze verdunning reageerden sommige niet-xanthomonaden ook met 2 van de 3 polyklonale antisera. Bij hogere verdunningen (1:900) verdwenen echter de meeste kruisreacties met de niet-xanthomonaden, maar ook de reacties met enkele stammen van *X. c.* pv. *campestris* en andere pathovars van *X. campestris*. Zes monoklonale antilichamen (MKA 17C12, 16B5, 20H6, 2F4, 18G12 en 10C5), geproduceerd tegen *X. c.* pv. *campestris* werden getest in "immunoblotting", een "enzyme immunoassay" (EIA), "dot-blot immunoassay" (DBI) en IF. De monoklonale antilichamen reageerden met het lipopolysaccharide (MKA 20H6, 2F4, 18G12 en 10C5) of met membraaneiwitten (MKA 17C12 en 16B5) van *X. c.* pv. *campestris* in "immunoblotting". Twee monoklonale antilichamen (MKA 17C12 en 16B5) reageerden met alle geteste xanthomonaden in DBI, maar niet in IF en EIA. De andere monoklonale antilichamen (MKA 20H6, 2F4, 18G12 en 10C5) reageerden in IF, EIA en DBI niet met alle stammen van *X. c.* pv. *campestris*,

maar nog wel met een aantal xanthomonaden, zoals *X. c.* pv. *vesicatoria* en *amoraciae*. Geconcludeerd werd dat de polyklonale antisera en een aantal monoklonale antilichamen gebruikt kunnen worden voor de identificatie van (een groep) *X. c.* pv. *campestris* stammen. De vraag in hoeverre de onderzochte monoklonale antilichamen en PKA 94 vals-negatieve en vals-positieve reacties kunnen geven bij het onderzoeken van zaadpartijen, wordt behandeld in hoofdstuk 5. Hiervoor werden in IF monoklonale antilichamen en een specifiek polyklonaal antiserum (PKA 94) gebruikt voor de detectie van *X. c.* pv. *campestris* in zaadextracten en voor de identificatie van verdachte kolonies vanaf uitplaatmedia. Het bleek dat de IF resultaten gedeeltelijk afhankelijk waren van de onderzochte zaadpartij, de gebruikte extractiemethode en monoklonale antilichamen. Dit bevestigde de al eerder gevonden interacties bij de uitplaatmethode. Het gebruik van het polyklonale antiserum resulteerde niet zonder meer in detectie van meer fluorescerende cellen in IF dan het gebruik van monoklonale antilichamen. In tegendeel, voor sommige zaadmonsters en extractiemethoden werden m.b.v. monoklonale antilichamen soms meer fluorescerende cellen waargenomen dan met het geteste polyklonale antiserum (PKA 94). De resultaten lieten zien dat het gebruik van twee methoden voor het extraheren van het pathogeen uit zaden (5 minuten en 2,5 uur schudden) wenselijk is om de kans op vals-negatieve resultaten in IF te minimaliseren. Wanneer monoklonale antilichamen in IF gebruikt werden voor de identificatie van kolonies bleken sommige *X. c.* pv. *campestris* kolonies vooral niet te reageren met MKA 16B5 en 17C12. Bovendien werd een kruisreactie van enkele saprofietische isolaten met MKA 17C12 gevonden. Op basis van deze studie werd MKA 20H6 beschouwd als het meest geschikte antilichaam voor de detectie en identificatie van *X. c.* pv. *campestris*.

Hoofdstuk 6 toont dat de correlatie tussen IF en uitplaten gelijk was voor MKA 20H6 en PKA 94. Naarmate meer cellen in IF gevonden worden, wordt de kans op isoleren van *X. c.* pv. *campestris* groter. Met IF worden doorgaans meer zaadmonsters positief gevonden dan met uitplaten. Het verschil en de overeenkomst tussen resultaten behaald met IF en uitplaten hangt voor een deel af van het onderzochte volume van het zaadextract in IF. Wanneer relatief grote volumina (b.v. 50 μl) onderzocht worden, zal de gevoeligheid van IF relatief groot zijn en de kans op vals-negatieve resultaten in IF klein

zijn. De correlatie tussen resultaten behaald met IF en uitplaten zal hierdoor echter afnemen. Geconcludeerd werd dat IF doorgaans een goede voorspelling geeft voor "gezondheid" van het zaad en een uitplaatmethode een goede voorspelling geeft voor "ziekte" of besmetting van het zaad.

Hoofdstuk 7 vat de problemen samen m.b.t. het gebruik van serologische technieken voor zaadovergaande bacteriën en geeft mogelijke oplossingen voor deze problemen.

Geconcludeerd wordt dat voor het toetsen van zaadpartijen op aanwezigheid van *X. c.* pv. *campestris* tenminste twee uitplaatmedia (b.v. NSCAA en CS20ABN) en twee extractiemethoden (5 minuten en 2,5 uur schudden van zaadmonsters bij kamertemperatuur) in IF en uitplaten gebruikt dienen te worden, teneinde het risico op vals-negatieve resultaten te minimaliseren. Voor IF is het aan te bevelen om zaadpartijen te onderzoeken m.b.v. verschillende monoklonale antilichamen, afzonderlijk of in een mengsel (b.v. MKA 20H6, 2F4 en 18G12), óf een monoklonaal antilichaam (b.v. MKA 20H6) en een specifiek polyklonaal antiserum (b.v. PKA 94).

Meer onderzoek is echter nodig om het belang van de invloed van storende saprofieten en antibacteriële stoffen op de detectie en overleving van *X. c.* pv. *campestris* in zaad te kunnen beoordelen.

Curriculum vitae

Antonius Adrianus Johanna Maria Franken werd op 13 december 1959 te Goirle geboren. In 1978 behaalde hij het Atheneum-diploma aan de Katholieke Scholengemeenschap Etten-Leur, waarna hij met zijn studie Planteziektenkunde aan de toenmalige Landbouwhogeschool te Wageningen begon. In 1984 studeerde hij af met als hoofdvakken fytopathologie, plantenfysiologie en moleculaire biologie. Daarna trad hij als bacterioloog/viroloog in dienst van het toenmalige Rijksproefstation voor Zaadonderzoek (RPvZ), dat via het Centrum voor Rassenonderzoek en Zaadtechnologie (CRZ) inmiddels is opgegaan in het Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO). Momenteel verricht hij binnen de afdeling Reproduktietechnologie, sectie Gezondheid, onderzoek naar de relatie tussen de vitaliteit en gezondheid van zaaizaden.

Nawoord

Bij deze wil ik graag allen bedanken die mij direct of indirect hebben geholpen bij het onderzoek en de totstandkoming van dit proefschrift.

Mijn promotor, Professor Dekker, ben ik erkentelijk voor de waardevolle adviezen, de getoonde belangstelling en het geschonken vertrouwen tijdens het onderzoek. Uw zeer vlotte en kritische reactie op alle versies (van onderdelen) van mijn proefschrift, heb ik zeer gewaardeerd.

Dr. Kees Langerak en Dr. Raoul Bino wil ik hartelijk danken voor hun continue interesse in de vorderingen van mijn proefschrift en voor de stimulerende gesprekken en adviezen. Verder wil ik mijn waardering uitspreken voor de ondersteuning die ik in de loop der jaren voor dit onderzoek op de Binnenhaven gehad heb van: Yvonne Birnbaum, Gé van den Bovenkamp, Janneke van Hateren, Miriam Poppelier, Marja Schippers, Jacoline Zilverentant en Patricia van der Zouwen. Patricia wil ik bovendien nog speciaal bedanken voor alle plak-, knip-, foto- en corrigeerwerk. Zonder de goede sfeer in de sectie Gezondheid, zou veel van dit werk niet hebben kunnen plaatsvinden. Ik wil alle collega's (incl. ex-collega's) bedanken voor het creëren van deze stimulerende sfeer. Het was en is steeds weer een waar genoegen om met jullie een borrel te drinken, te praten over eten (dit voert tegenwoordig nogal eens de boventoon), film en sport (sportonderwerpen komen de laatste tijd overigens wel wat in de verdrukking). Soms is het wel even knokken om een stukje leverworst te bemachtigen, maar over het algemeen kunnen we prima met elkaar opschieten.

Collega's van de NAKG, NAKS en zaadbedrijven ben ik erkentelijk voor hun betrokkenheid bij dit onderzoek. Zij zijn niet alleen betrokken geweest bij de initiatie van dit onderzoek, maar ook hebben zij praktisch en theoretisch bijgedragen aan dit onderzoek. Hartelijk dank voor de goede en open samenwerking. Het Laboratorium voor Monoklonale Antistoffen (LMA) wil ik bedanken voor de prettige samenwerking. Zonder de goede ideeën en praktische ondersteuning van het LMA was dit onderzoek niet mogelijk geweest.

Tevens is dit voor mij een aanleiding om alle co-auteurs van de artikelen opgenomen in dit proefschrift hartelijk te bedanken voor hun bijdrage.

De sectie Tekstverwerking van CPRO-DLO wil ik bedanken voor hun vlotte hulp bij het "camera-ready" maken van dit proefschrift.

Alle nog niet genoemde collega's en vakgenoten van zowel binnen als buiten CPRO-DLO wil ik bedanken voor hun positieve en kritische discussies over de onderwerpen behandeld in dit proefschrift.

Ik wil tevens alle directies (RPvZ, CRZ en CPRO-DLO) die ik in de loop van de jaren heb meegemaakt, bedanken voor hun medewerking en het beschikbaar stellen van de benodigde faciliteiten.

Vermeld dient ook te worden dat dit onderzoek gedeeltelijk gefinancierd werd uit gelden beschikbaar gesteld voor het verbeteren van keuringsmethoden door de toenmalige directie VKA (Voedings- en KwaliteitsAangelegenheden) van het Ministerie van Landbouw, Natuurbeheer en Visserij.

Mijn familie en vrienden wil ik hartelijk danken voor het feit dat zij hebben bijgedragen aan mijn behoefte om zo nu en dan eens over andere zaken te praten dan *Xanthomonas campestris* pv. *campestris* (overigens een zeer interessante bacterie).

En natuurlijk wil ik als laatste mijn steun en toeverlaat, Adrie, bedanken, die me niet alleen geholpen heeft met het kritisch beoordelen en verbeteren van mijn manuscripten, maar ook met het opknappen van klusjes in en rond huis die ik nogal eens liet zitten. Ik kijk uit naar de dag dat we samen weer het gras kunnen maaien.