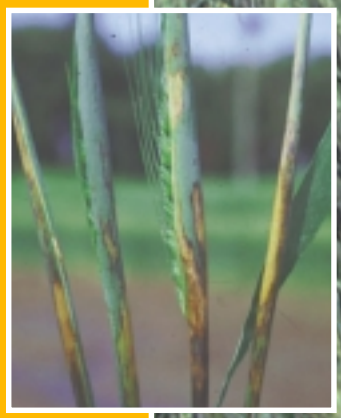
The Bacterial Diseases of Wheat

Concepts and Methods of Disease Management



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CIMMYT
Sustainable Maize and Wheat Systems for the Poor

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Concepts and Methods of Disease Management

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Contents

Abstract

Duveiller, E., L. Fucikovsky, and K. Rudolph, eds. 1997. *The Bacterial Diseases of Wheat: Concepts and Methods of Disease Management*. Mexico, D.F.: CIMMYT.

The most common bacterial pathogens that attack wheat are grouped under the names *Xanthomonas translucens* and *Pseudomonas syringae.* Pathovars (bacterial strains pathogenic to a specific host or hosts) included in *X. translucens* can cause significant crop losses, while the ones grouped under *P. syringae* may severely affect the grain quality of wheat and are widely distributed in many temperate and subtropical wheat-growing regions. There is also a range of other bacterial diseases that have a more limited distribution or may cause crop losses just in certain localities.

All of these are the subject of the present manual, which focuses on identifying and managing wheat bacterial diseases. It presents concepts and methods that will help those not familiar with bacterial diseases to correctly identify and diagnose them.

Despite numerous reports of bacterial diseases on wheat worldwide, the study of bacterial diseases on wheat and triticale has been limited, and information on how to handle them is rarely available. Managing bacterial diseases is somewhat complex and often requires an integrated approach combining adequate control measures and breeding for genetic resistance. The authors of this manual have therefore included general principles and guidelines to help breeders and pathologists to both control these diseases and select for genetic resistance.

Other Manuals in This Series

Stubbs, R.W., J.M. Prescott, E.E. Saari, and H.J. Dubin. 1986. *Cereal Disease Methodology Manual.* Mexico, D.F.: CIMMYT.

Eyal, Z., A.L. Scharen, J.M. Prescott, and M. van Ginkel. 1987. *The Septoria Diseases of Wheat: Concepts and Methods of Disease Management.* Mexico, D.F.: CIMMYT.

Roelfs, A.P., R.P. Singh, and E.E. Saari. 1992. *Rust Diseases of Wheat: Concepts and Methods of Disease Management*. Mexico, D.F.: CIMMYT.

Wilcoxson, R.D., and E.E. Saari, eds. 1996. *Bunt and Smut Diseases of Wheat: Concepts and Methods of Disease Management.* Mexico, D.F.: CIMMYT.

Preface

Fundamental to CIMMYT's mission to improve wheat productivity in developing countries while protecting the environment is the effort to combat diseases that reduce wheat yields in farmers' fields. CIMMYT's primary strategy for keeping diseases in check with minimal environmental effects is to incorporate genetic disease resistance into the improved germplasm it develops. However, knowing how to identify, manage and control diseases when they occur is essential to maintaining and increasing wheat production levels. For this reason, over the years the CIMMYT Wheat Program has generated and distributed a series of technical manuals that help identify wheat diseases and describe sustainable methods for their control.

Targeted to a developing world audience, this practical series has focused on methods and information that are particularly useful to national program researchers and extension workers in developing countries. The manuals describe concepts and techniques ranging from basic to advanced and thus can effectively aid researchers with varying degrees of phytopathological expertise to manage wheat diseases.

This, the most recent manual in the series, brings together under one cover widely dispersed information on the bacterial diseases of wheat. These diseases are often considered difficult to manage and may be hard to identify, since their symptoms are sometimes confused with those of physiological or stress-induced damage. Another complication is that most pathogenic bacteria are of similar size and shape, and thus cannot be identified based solely on microscopic observation; consequently, the methods used for isolating and identifying these pathogens are very different from those used on other pathogens. To help readers avoid these pitfalls, this manual includes procedures specifically for detecting and verifying the presence of bacterial pathogens.

CIMMYT is grateful to the five outside experts in cereal bacteriology who contributed to the preparation of this manual: Klaus Rudolph, Jan von Kietzell, Claude Bragard, Henri Maraite, and Leopold Fucikovsky. They worked in coordination with CIMMYT wheat pathologist Etienne Duveiller, who also co-authored three of the four chapters in the manual. As a result of their cooperation, agricultural scientists in the developing world have a valuable resource to aid them in their work.

This preface would not be complete without mentioning the numerous parties who cooperated in producing this publication. CIMMYT wishes to acknowledge the contribution of the Belgian Administration for Development Cooperation (BADC), without whose support this manual would not have been possible. We also wish to express our appreciation to Lu Piening, A.K. Vidaver, R.L. Forster, A. Mavridis, J. Annone, A. Fessehaie, S. Oba, A.M. Sánchez and K. Rudolph for the use of their photographs. We are grateful to Miguel Mellado and Eliot Sánchez for designing the manual and preparing the drawings, and to Sarah Fennell for reviewing the manuscript.

Finally, a word on the potential usefulness of the manual. Aside from the extensive literature review (more than 300 references) provided on the subject of bacterial diseases of wheat, the authors have included recipes for general-purpose and specific culture media as well as a glossary listing words used in the manual. Explanations of bacteriological procedures are enhanced by the use of drawings, step-by-step instructions, tables, graphs, and / or color photographs. The combined effect of all these elements has resulted in a manual of unusual clarity and ease of use which we hope will find a warm welcome among people working in the field in both developing and industrialized countries.

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Introduction

This manual furnishes the general concepts, methods and background information for diagnosing wheat (*Triticum aestivum* L.) bacterial diseases, as well as specific descriptions and methodologies that help identify a number of the more important bacterial pathogens. It is meant to aid workers who may encounter the bacterial diseases of wheat and need to identify them without the help of a pathologist and with limited laboratory facilities. For this reason, step-by-step instructions for performing simple tests in basic laboratory conditions are provided in each chapter.

A manual meant to help diagnose disease problems would not be complete if it did not also provide information on how to control them. Managing the diseases bacterial pathogens cause is somewhat complex and often requires an integrated approach involving disease control measures and breeding for genetic resistance. The authors have therefore given general principles and guidelines that should help breeders and pathologists to both control bacterial diseases and select for genetic resistance. As an additional aid, they have included the commonly observed symptoms and main areas of distribution of bacterial diseases that occur naturally on wheat and triticale (X *Triticosecale* Wittmack), a stable cross between wheat and rye (*Secale cereale* L.), based on reports of their natural incidence on these hosts (see **Table**).

An increasing number of bacterial disease outbreaks, sometimes caused by new pathogens, has been reported on wheat in widely varying geographical areas. This may be due to several factors: 1) more research is being conducted on bacteria (making the identification of bacterial diseases more likely), 2) germplasm exchanges have increased and as a result there is a heightened risk of disease spread through contaminated seed, 3) wheat is expanding into more disease-prone environments (i.e., warmer and more humid areas), 4) mercury seed treatments (which were highly effective but also highly toxic) are no longer utilized, and 5) the occurrence in limited regions of unusual climatic conditions favors "new" bacterial species (the "new" disease has probably occurred before, but has never been reported).

Despite numerous reports of bacterial diseases on wheat worldwide, the study of bacterial diseases on wheat and triticale has been limited, and quantitative information—for example, on crop losses and disease epidemiology—is rarely available. This is partly the result of the sporadic incidence of these diseases on wheat, their distribution in specific agroecological areas and the difficulty of conducting the long term experiments needed for understanding their epidemiology. It should also be noted that while most fungal pathogens are isolated, examined and identified by general plant pathologists, bacterial diseases are often left to the specialist or ignored. In addition, reports on the occurrence of bacterial diseases on wheat are sometimes based on symptom observation only, without isolation of the causal pathogen or confirmation of its identity through appropriate testing.

In recent years, plant bacteriology has benefited from the rapid development of new technologies, particularly molecular methods, that are quickly becoming indispensable for taxonomic and identification purposes. Albeit unintentionally, these developments have widened the technological gap between specialized pathologists and extension agents or researchers working in the field. Also, since highly advanced technologies are for the most part unavailable in developing countries, there is an urgent need for relatively simple and reliable diagnostic and identification methods that can be used in technologically limited conditions.

In response to this perceived need, the authors have put together this manual, which in the first chapter introduces the basic principles involved in diagnosing and identifying the bacterial pathogens of wheat. Chapter 1 also provides a selection of simple tests that can be used in laboratories with

Bacterial diseases naturally found on wheat: their symptoms, main areas of distribution, and some key references.

Bacterium and author citation	Disease	Distribution	Selected references[a]
Diseases caused by *Xanthomonas translucens* pathovars			
Xanthomonas translucens pv. *undulosa* (Smith, Jones and Reddy 1919) Vauterin, Hoste, Kersters and Swings 1995	Leaf streak, leaf stripe, black chaff	Worldwide, Brazil, Mexico, Pakistan, USA, China	Duveiller (1994b) Mohan and Mehta (1985) Bamberg (1936) Akhtar and Aslam (1986) Smith (1917) Sun and He (1986)
Closely related pathovars : *Xanthomonas translucens* pv. *cerealis* (Hagborg 1942) Vauterin, Hoste, Kersters and Swings 1995; *Xanthomonas translucens* pv. *translucens* (Jones, Johnson and Reddy 1917) Vauterin, Hoste, Kersters and Swings 1995; *Xanthomonas translucens* pv. *secalis* (Reddy, Godkin and Johnson 1924) Vauterin, Hoste, Kersters and Swings 1995			
Diseases caused by *Pseudomonas syringae* pathovars			
Pseudomonas syringae pv. *syringae* van Hall 1902	Leaf blight	Worldwide, USA	Shane and Baumer (1987) Otta (1974)
Pseudomonas syringae pv. *atrofaciens* (McCulloch 1920) Young, Dye and Wilkie 1978	Basal glume rot	Europe, New Zealand, Mexico, Syria	Wilkie (1973)
Pseudomonas syringae pv. *japonica* (Mukoo 1955) Dye et al. 1980	Black node, blight, stripe	Japan	Mukoo (1955)
Other bacterial diseases			
Bacillus megaterium pv. *cerealis* Hosford 1982	Tan streak, white blotch	USA	Hosford (1982)
Clavibacter iranicus (ex Sharif 1961) Davis, Gillaspie, Vidaver and Harris 1984	Gumming	Iran	Carlson and Vidaver (1982a) Scharif (1961)
Clavibacter michiganensis subsp. *tessellarius* (Carlson and Vidaver 1982) Davis, Gillaspie, Vidaver and Harris 1984	Mosaic	USA	Carlson and Vidaver (1982b)
Clavibacter tritici (ex Hutchinson 1917) Davis, Gillaspie, Vidaver and Harris 1984	Tundu, yellow ear rot	India, China, Ethiopia, Egypt	Paruthi and Gupta (1987)
Erwinia rhapontici (Millard 1924) Burkholder 1948	Pink seed	USA, Canada, UK, France, Israel	Roberts (1974) Diekmann and Putter (1995)
Pseudomonas cichorii (Swingle 1925) Stapp 1928	Stem, head melanosis	Canada	Piening and MacPherson (1985)
Pseudomonas fuscovaginae (ex Tanii, Miyajima and Akita 1976) Miyajima, Tanii and Akita 1983	Brown sheath rot	Mexico	Duveiller and Maraite (1990)

[a] See Bradbury (1986) for more references and details.

no more than the basic equipment. Mainly for the purpose of providing information and contributing in some small way to bridging the technological gap, the authors give a brief overview of the range, uses and limitations of several molecular methods based on nucleic acid hybridization. They also recognize that though they may not have the facilities nor the training required to apply more advanced procedures, most readers wish to stay abreast of the latest techniques being applied in plant bacteriology.

Among the bacteria that infect wheat, only *Xanthomonas translucens* pathovars are able to cause significant crop losses, especially in warm, humid climates. Due to the importance and broad distribution of *X. translucens* pv. *undulosa*, particular attention is given to this pathovar in Chapter 2. *Xanthomonas translucens* pv. *undulosa* causes bacterial leaf streak or stripe (BLS), known as black chaff when found on the glumes (Smith *et al.* 1919). The distribution of BLS is worldwide (Duveiller 1989), and triticale seems to be particularly susceptible to it (Cunfer and Scolari, 1982).

The third chapter of this manual focuses on the *Pseudomonas syringae* group, which includes several widely distributed pathovars that attack wheat. Wheat pathogens of the *P. syringae* group occur in maritime as well as temperate continental climates and have been reported from nearly all temperate and subtropical wheat-growing regions. Bacteria of the *P. syringae* group have not been studied thoroughly, probably because they occur sporadically in spring or summer under extremely humid conditions. Although the diseases these pathovars cause are generally considered to be of minor importance, some can severely reduce the grain quality of bread wheat.

A range of other bacterial diseases are infrequently found on wheat (Bradbury 1986), have a more limited distribution or are reported in locations where specific conditions are present. These diseases are described in the fourth and last chapter of this manual, which also presents several lesser known pathogens that are not well documented but may cause crop losses or constitute production constraints in certain localities.

The names used in this manual are those included in the International Society of Plant Pathology's official list of plant pathogenic bacteria (Dye *et al.* 1980), amended by Young *et al.* in 1991. The list was updated by Young *et al.* in 1996 but considered only for *X. translucens* in this manual. Recipes for general purpose media, as well as more specific growth media, are given in the Appendix at the end of the manual. Finally, the authors have included a lengthy bibliography as an aid to those interested in delving more deeply into this area of research. It is their hope that the manual, in all its parts, will prove to be as a useful tool as the previous manuals in this series.

Chapter 1 : General Concepts and Methods for the Identification of Pathogenic Bacteria of Wheat

E. Duveiller, C. Bragard, K. Rudolph and L. Fucikovsky

This chapter presents the general concepts, procedures and principles for identifying bacterial diseases on wheat. The methods reviewed cover from symptom observation and bacteria isolation to pathogen characterization. Pathogens can usually be characterized by conducting biochemical tests. However, further characterization is possible by determining the pathogen's host range or by using serological or molecular techniques.

Although time consuming, conducting appropriate differential tests and fulfilling Koch's postulates (Agrios 1978) is still the easiest and cheapest way to detect and identify plant pathogenic bacteria. While many biochemical and physiological determinative tests have been developed to characterize bacterial pathogens based on phenotypic features, not all of them have the same taxonomic value, and results obtained with some of them may vary among a population of strains within a species. However, a few tests are generally sufficient to identify certain groups or genera of microorganisms. Thus only a small number of tests covering the key differences among wheat pathogenic species is presented in this manual. For example, only one test is needed for distinguishing *Erwinia rhapontici* from other wheat pathogens, based on the pathogen's ability to grow anaerobically in a medium amended with glucose. Similarly, fluorescent plant pathogenic pseudomonads may be identified using a determinative scheme known as LOPAT reactions (Lelliott *et al.* 1966). It should be noted, however, that more tests are necessary to do a complete description of a bacterial species. For detailed taxonomic information and results of determinative characters, see Krieg and Holt (1984), Bradbury (1984; 1986), and Klement *et al.* (1990a).

In recent years, the study of plant pathogenic bacteria has benefited from new biotechnological methods. The development of monoclonal antibodies has significantly improved serological techniques. Most importantly, techniques based on nucleic acid analysis have made it possible to do more precise taxonomic studies, as well as studies of factors controlling pathogenicity. In contrast to conventional methods that rely on pathogen induced reactions, these emerging techniques have the enormous advantage of characterizing, identifying, and detecting microorganisms based on genomic information. Although these technologies are not yet widely available, new developments and future prospects arising from studies currently being conducted on wheat pathogenic bacteria are briefly presented in this chapter.

Figure 1.1. Water-congested green tissue (water-soaking) on a wheat leaf may be an early symptom of bacterial leaf streak.

Disease Symptoms

Symptoms caused by bacterial diseases may vary greatly, depending on the pathogens involved. A bacterial origin is suggested by water-soaked spots or water-soaked margins around lesions consisting of water-congested green tissue (**Figure 1.1**) in the early stages of infection. The lesions are greasy and translucent in appearance and may produce an exudate. An exudate consists of droplets of bacterial slime emerging from the leaf surface through natural openings (stomata, hydathodes). They are typical of bacterial leaf streak (BLS) caused by "*Xanthomonas translucens*" (a broad term used in this manual to refer to the group of pathovars that cause BLS) and can be observed better in the morning or after rain, when air humidity is high.

It may be useful to compare observed symptoms to pictures in reference manuals (Wiese 1987, Zillinsky 1983); recording them will save a lot of time when doing other identifications. However, bacterial diseases should never be identified based on symptoms only, unless the disease has previously been identified by an experienced investigator in a region where he knows environmental conditions very well and where outbreaks are frequent. As a rule, the pathogen must be isolated; once isolated, it must be shown to be pathogenic on the host. The precise

species is definitively identified through a set of appropriate biochemical and physiological tests or using molecular tools.

Microscopic Observation

Microscopic observation can help determine whether an unknown disease is of bacterial origin. Unlike fungal pathogens, wheat pathogenic bacteria cannot be identified based solely on their morphology. All wheat pathogenic bacteria are rod shaped and measure 0.8-1.5 x 2.0-2.5 µm on average. Fresh samples collected from the field should first be observed under the microscope for an indication that the lesion may be caused by a bacterium. Since bacterial cells in these samples are living and hyaline, they cannot be observed under a transmitted light microscope. Dark field microscopy provides very high contrast and should be used to detect transparent bacterial cells oozing from diseased tissue. In the case of *Xanthomonas translucens* pv. *undulosa* or *Pseudomonas syringae*, a small piece of plant tissue cut from the edge of the lesion, where living bacteria are more abundant, will typically show a white smear of bacteria oozing from the edge of the sample **(Figure 1.2)**. If a dark field microscope is not available, a pseudo-dark field can be obtained using a phase contrast microscope. On a Zeiss microscope, this is achieved with a x10 objective (Ph1) and the condenser set on 3. Bacteria can be observed in more detail with a phase contrast microscope (x40 magnification; Ph2) and the condenser set on 2. With few exceptions, failure to detect large numbers of bacteria oozing from diseased material indicates either that the disease is not caused by a bacterium or that the wrong part of the specimen has been examined.

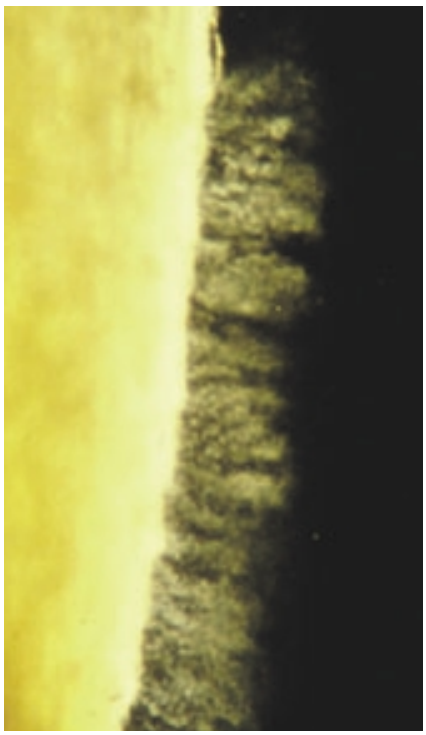


Figure 1.2. White masses or streams of *Pseudomonas syringae* cells oozing from the edge of a wheat sample observed under a dark field microscope.

To make a correct diagnosis, it is important to observe a sample of diseased tissue taken from the edges of the lesion, since rotted or necrotic tissue may harbor significant amounts of saprophytic bacteria, which could be misleading. If oozing is clearly observed and there is no obvious association with fungi, the isolation procedure should be initiated.

Isolation

Choosing a culture medium

Wheat pathogenic bacteria grow well on most general media. The use of semi-selective media may be helpful, but the importance of their selectivity is often overestimated. They are usually not necessary for isolating a bacterium from a lesion, but are recommended for testing seed for bacterial pathogens. The more sugar in the medium, the faster the growth; however, undesirable saprophytic bacteria, often present in the sample, may develop so fast that their colonies may cover those originating from pathogenic bacteria.

If a fluorescent *Pseudomonas* is suspected, King's medium B (KB) (King *et al.* 1954) should be used (see Appendix). On this general medium, the genus produces a yellow fluorescent pigment (pyoverdine) that is better observed under near-ultraviolet light **(Figure 1.3 a and b)**. KBC agar (see Appendix) is a modification of KB useful for isolating *P. syringae* pv. *syringae* (Mohan and Schaad 1987). To isolate *P. syringae* pvs. *syringae* and *atrofaciens* from wheat, the KBC medium should be modified by reducing the concentration of boric acid from 1.5 g/L to 0.5 g/L (Fessehaie 1993; see Chapter 3 this manual). Water quality is important because fluorescein production may be hampered by the presence of iron (Fe^{+++}). Also, pH may need to be adjusted to 7.2, particularly with some agar brands. Best results are obtained with Difco proteose peptone No. 3.

Non-fluorescent bacteria will grow on KB medium but can also be cultured on three other commonly used general purpose media: nutrient agar, YPGA and Wilbrink's medium (see Appendix). Growth is somewhat slower on nutrient agar, which can be an advantage as slower saprophytic growth will make it easier to spot colonies of a putative pathogenic

species. Another advantage is that this medium is available commercially.

The medium XTS (see Appendix), a modification of nutrient agar, has been suggested for isolating *X. t.* pv. *translucens* from cereals (Schaad and Forster 1985), but gentamycin-related problems may sometimes occur (Schaad and Forster 1989), making WBC agar a good alternative (Duveiller 1990b) (see Appendix).

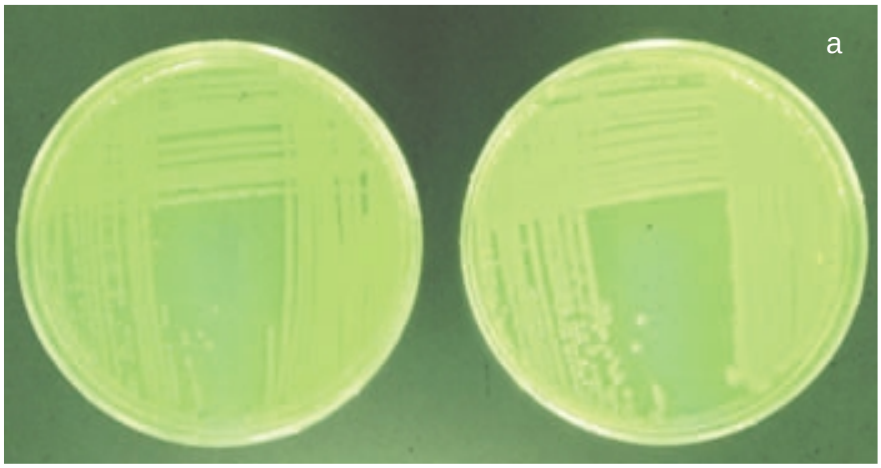
The agar surface must be dry when using these media for isolation because moisture will not allow the development of individual colonies. Condensation in the Petri dishes is reduced if agar is poured at 46°C (flasks should be kept in a waterbath for 20 min at 46°C before pouring) and the dishes are stacked for 24-36 h before using. If there is free water on the agar, the dishes should be dried under laminar flow for 20 min or by placing them open and upside down in an incubator for 30 min at about 50°C.

Isolating the bacterium

Although not usually necessary, plant tissue can be externally disinfected by soaking in bleach at 1% active sodium hypochlorite for 3 min, followed by rinsing in sterile distilled water.

A drop of sterile distilled water is pipetted onto a empty, sterile Petri dish. Plastic dishes are preferred because drops of water remain globular on the bottom of the plate due to weaker surface tension. This facilitates preparation of several macerates in different areas of the same Petri dish.

Remove a small piece (about 3 x 3 mm) of plant tissue with a flamed scalpel from the edge of the lesions, at the limit between healthy and diseased tissue, and aseptically place it in the drop of water in the Petri dish. Beginners tend to macerate pieces of plant material that are too large (Rudolph *et al.* 1990). Cut up the sample using a scalpel and forceps. Put the lid back on the dish and macerate for 10-15 min so bacteria are released into the water. Using a sterile loop, take an aliquot of the suspension and streak it onto a fresh agar plate starting from the edge and zig-zag across a fourth of the agar surface. After streaking the first field, flame the loop, cool it down in alcohol and briefly flame it again to eliminate excess alcohol. Turn the dish about 90° and streak again, making sure to



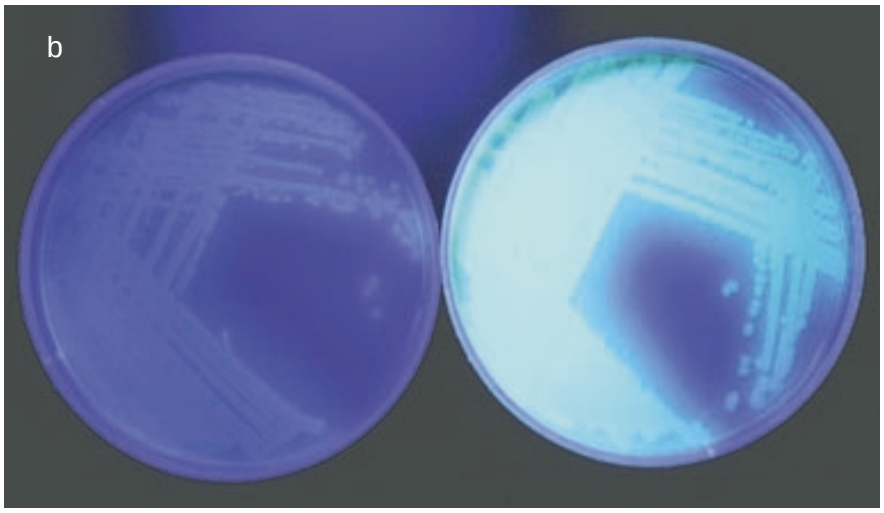
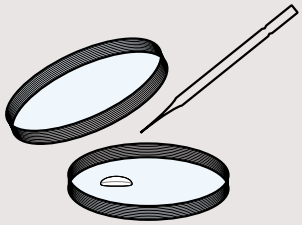
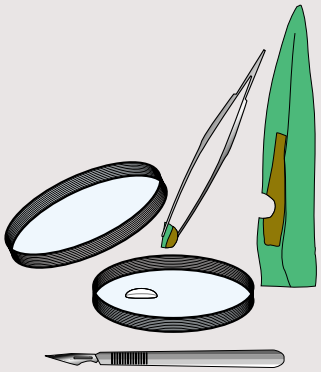


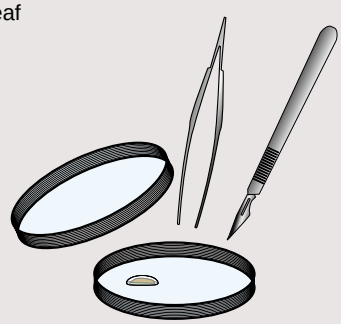
Figure 1.3 a. Fluorescence induced on King's medium B by a pure culture of *Pseudomonas fuscovaginae* (left) and by a common saprophyte on wheat plants, *P. fluorescens* (right); plates observed under normal lighting.
b. Observation under near U.V. light of fluorescence induced on King's medium B by a pure culture of *P. fuscovaginae* (left) and by *P. fluorescens* (right).



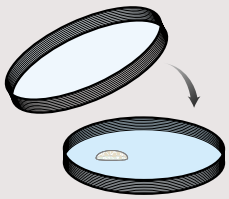
1 Put a drop of sterile distilled water in a empty, sterile Petri dish



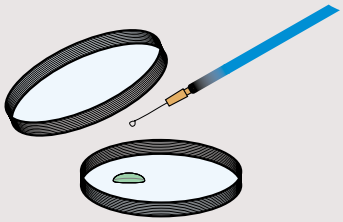
2 Cut a small piece of leaf tissue from the edge of the lesion and put it in the drop of water



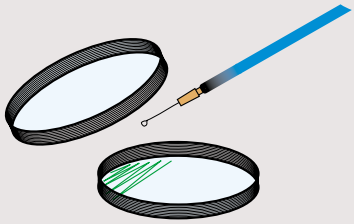
3 With the help of forceps and scalpel crush the sample in the drop of water



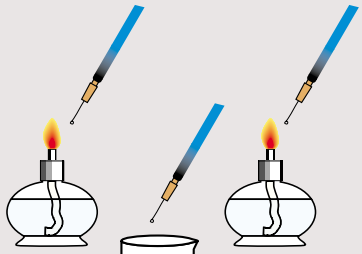
4 Let macerate for 10-15 minutes



5 With a sterile loop take an aliquot from the macerate



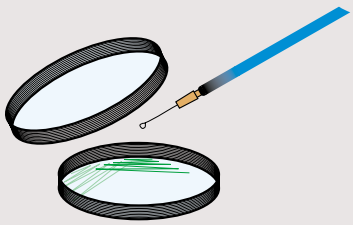
6 Streak (zigzag) the first field on a fresh agar plate



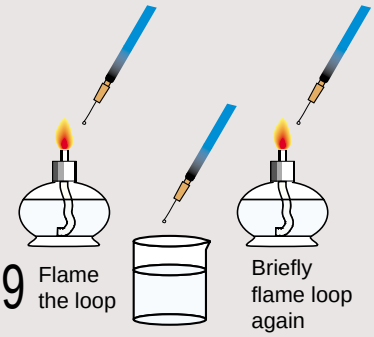
7 Flame the loop

Dip it in alcohol

Briefly flame loop again



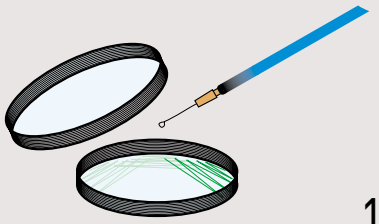
8 Streak the second field



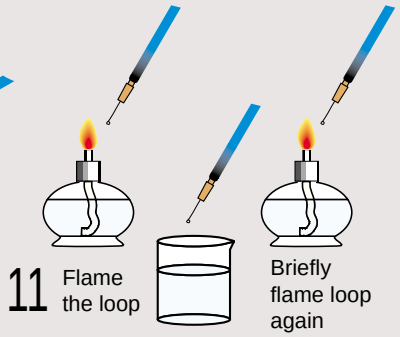
9 Flame the loop

Dip it in alcohol

Briefly flame loop again



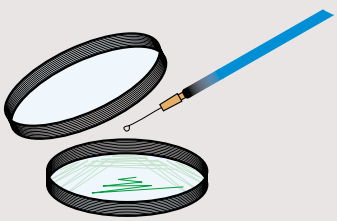
10 Streak the third field



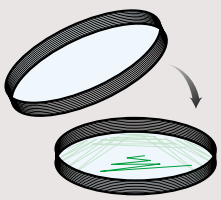
11 Flame the loop

Dip it in alcohol

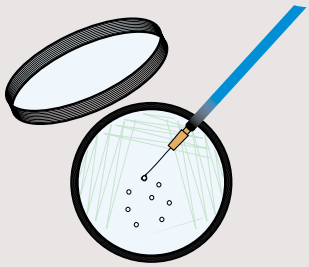
Briefly flame loop again



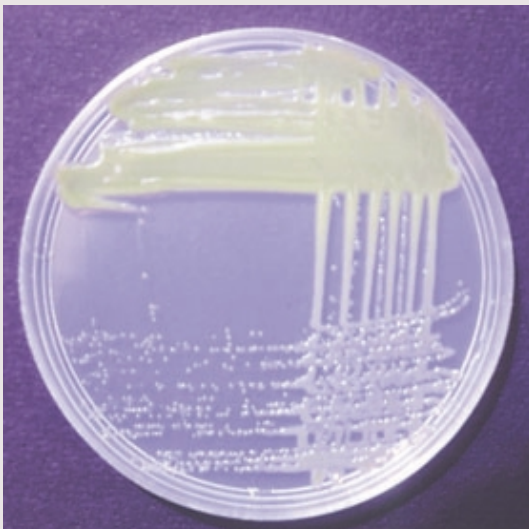
12 Streak the fourth field



13 Incubate the Petri dish upside down at ± 28ºC for 3-5 days



14 Subculture single colonies on fresh plates to obtain pure cultures



overlap with the edge of the first field. Turn the dish 90° and streak two more times, flaming and cooling the loop each time. When streaking the fourth field, overlap with the edge of the third field, but be careful not to touch the first (**Figure 1.4**). Plates should be incubated upside down to keep water from dripping on the agar.

Incubation

Most pathogenic bacteria grow well at 27°C, but "*X. translucens*" grows faster at 30°C. Plates are usually incubated for 36-48 h before subcultures are made. Subculture single colonies of the most abundant colony type by streaking onto new agar plates, in different fields, as described above (Figure 1.4). If the bacterium is not known, different putative colonies should be cloned. If the colony's appearance is specific to a given species, it should be recorded as an identifying character. Colonies can be smooth, convex or show a particular color.

Pathogenicity Tests

Before attempting characterization tests to identify the pathogen, pathogenicity should be tested on a susceptible host and Koch's postulates verified, as follows. Healthy plants of the host on which the disease appeared are inoculated with a pure culture of the suspected pathogen, which should induce the same symptoms. The pathogen is re-isolated in pure culture, where it must show exactly the same characteristics as the strain originally isolated from the plant (Agrios 1978).

For this pathogenicity test, healthy susceptible host plants produced in the greenhouse are inoculated with large amounts of bacteria. Usually the wheat stem or a leaf is pricked with a needle that has passed through fresh pure culture (24-36 h) of the suspected bacterium. For leaf infiltration or inoculum spray (**Figures 3.8 and 3.9**, Chapter 3), a water suspension at a concentration 10^6 - 10^7 bacterial cells per ml (colony forming units, or cfu) should be adequate to show pathogenicity. The concentration can be adjusted with a Petroff-Hausser counting chamber (**Figure 1.5**), a spectophotometer or a colorimeter (i.e. Klett-Summerson) to an optical density of 0.06, which has to be diluted to 1:10 or 1:100.

If the bacterium is pathogenic, after several days of incubation under high moisture conditions the inoculated sample appears water-soaked and then a lesion similar to the symptom is observed. Water-soaking, however, is not typical of *P. syringae* pv. *atrofaciens*. Results are usually better if temperature is not too high (20-24°C). High humidity causes dew to form on the plant. Free water on the plant surface can be obtained by covering the pot containing the inoculated plant with a plastic bag and sealing it around the pot with a rubber band **(Figure 1.6)**. A bigger plastic moisture chamber can also be constructed with an ultrasonic humidifier as the source of moisture. The advantages of this system are that the delicate cool water mist can be regulated using a timer **(Figure 1.7)** and more pots can be used at one time.

Lesions extending several centimeters from the inoculation point on the host plant should appear after about five days' incubation if the test is positive (host/bacterium compatibility). However, the bacteria should be re-isolated from the border of the lesion to confirm pathogenicity. If the inoculated bacterium is not pathogenic, a hypersensitive reaction (no compatibility) limited to the inoculation point is observed, and the plant tissue collapses within 24 h.

Physiological and biochemical determinative tests help identify the pathogen. They can be carried out in most plant pathology laboratories. In recent years, quick molecular tools such as DNA fingerprinting have increased the precision of bacterial characterization, but these tools are not yet widely available. It is now generally accepted that only polyphasic approaches based on phenotypic, chemotaxonomic and genotypic data produce stable, general purpose classifications. DNA homology has become the ultimate criterion for defining bacterial species (Vauterin *et al.* 1993); determinative tests that reveal the bacterial phenotype through a selected set of differential reactions may not always distinguish among bacteria (Dye 1962).

Most plant pathogenic bacteria are host specific or attack a limited number of plant species belonging to the same group (Lelliott and Stead 1987); this specificity provides a way to identify as distinct pathovars bacteria that give similar results on determinative tests. As an example, *X. campestris* can be identified based on determinative tests. However, this species includes more than 60 pathovars. Similarly, in the *X. translucens* group, four pathovars have been characterized as pathogenic on wheat and other cereals based on host range.

Figure 1.4. Standard procedure for isolating plant pathogenic bacteria. Inset: Agar plate showing a purified culture of *Xanthomonas translucens* pv. *undulosa* growing on Wilbrink's medium.

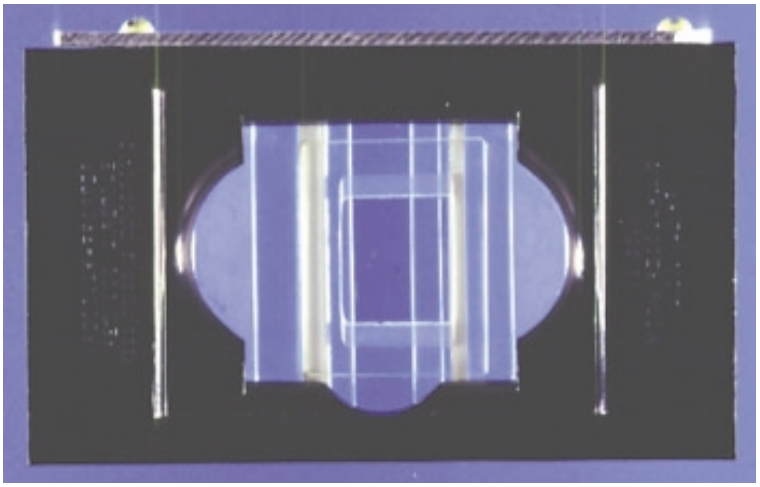


Figure 1.5. Petroff-Hausser counting chamber with a reinforced glass cover that allows observation under phase contrast microscope. To estimate bacterial cell concentration, dilute the bacterial suspension 1:200 and make a count of 10-20 squares under the phase contrast microscope (x400). Bacteria/ml = (total bacteria counted x 200 x 20 10^6)/number of small squares counted. (Photo: A.M. Sánchez.)

Figure 1.6. Humid chamber made by covering pots and wheat plants with a plastic bag to induce symptoms caused by a pathogenic bacterium.

Figure 1.7. Ultrasonic humidifier modified to supply a cool mist of water in a plastic incubation chamber.

Pathogen Characterization Using Simple Methods

This section describes a small number of tests that are sufficient to decide: 1) whether a given bacterial culture is likely to be the suspected wheat pathogen, and 2) which organisms of those isolated from a diseased plant need further study. Among these tests, the following give a quick clue of the micro-organism involved: Gram test, heat test for spores, production of fluorescent pigment, acid production from glucose, Kovacs' oxidase and nitrate reduction. Other tests for a more precise identification of wheat pathogenic bacteria are also presented. The tests are discussed roughly in the same order in which they should be conducted, although several of them can be done simultaneously, with the same culture.

Characterization tests are done on pure cultures (strains) grown after cloning single colonies from previously isolated bacteria. To save time and money, tests should be done on strains that have proved pathogenic on the susceptible host. Characterization tests should be done on fresh pure cultures only, and comparisons between strains should be done on young cultures of the same age (ideally 24-36 h old). There are many determinative tests (Schaad 1988a), of which only the taxonomically valuable ones that help to confirm or discard a hypothesis are necesary (see **Table**). When conducting these tests, it is very important to include a control strain, i.e., a reference strain or one with a known reaction to a particular test. A non-inoculated tube should be kept as a reference for assessing color changes induced by the bacterium in inoculated tubes.

Several determinative tests have recently become commercially available (API-20, BIOLOG MicroPlate™ system). Although easy to use, these standardized, miniaturized, multi-test systems are not always specific for identifying wheat pathogens, particularly when identification is based on the pathovar concept. Besides being costly, they have an expiration date, must be stored properly, and are not easily shipped to some destinations.

Gram test

The Gram reaction is one of the first tests to be conducted because it separates bacteria into two fundamentally different groups based on their wall properties. This test is based on staining with different dyes. If the primary dye—a complex of crystal violet and iodine—is retained, the bacterium appears blue or dark purple under the microscope and is Gram positive. If the counterstain (safranine) is retained, bacteria appear red/pink under the microscope and are Gram negative. Described below is the classic procedure for performing the Gram test and Ryu's method, which is much faster.

Standard staining procedure. For this procedure (adapted from Bradbury 1970), make a cloudy suspension using bacteria from a fresh agar plate and sterile water. Prepare a smear on a glass slide (previously wiped with alcohol) and let it dry. After 10 minutes, fix the bacteria by passing the bottom of the slide two or

Differential reactions induced in determinative tests by bacterial species naturally found on wheat.

Determinative tests	Bacterial species: *X. translucens* pv. *undulosa* (group *translucens*)	*P. syringae*	*P. cichorii*	*P. fuscovaginae*	*C. m.* subs. *tessellarius*	*C. tritici*	*C. iranicus*	*E. rhapontici*	*B. megaterium*	*P. fluorescens*
Gram	–	–	–	–	+	+	+	–	+	–
Heat test for spores	–	–	–	–	–	–	–	–	+	–
Fluorescence	–	+	+	+	–	–	–	–	–	+
Kovacs' oxidase	–	–	+	+	–	–	–	–	–	+
Strictly aerobic	+	+	+	+	+	+	+	–	+	+
Acid from: trehalose	+			+				+		+
inositol	–	+		–				+		
sorbitol		+		–				+		+
Nitrate reduction	–	–	–	–						+
Levan production	–	+	–	–						
2-ketogluconate production	–	–	–	–						+
Arginine dihydrolase	–	–	–	+						+
Potato soft rot	–	–	–	–	–	–	–	+	+	+
Esculin hydrolysis	+									
Pathogenicity on wheat	+	+	+	+	+	+	+	+	–	–
Tobacco hypersensitivity	(+)	+	+	+					–	–

three times over a flame. Cover the slide for 30 sec with 0.5% aqueous crystal violet. Pour off and wash with 1% iodine solution in 2% aqueous KI solution. Wait 30 sec and wash with ethanol until the color stops running. Rinse with water. Counterstain with 0.5% aqueous safranine for about 3 min. Rinse again with water and let dry. Add immersion oil and observe under the microscope (x1000). Gram positive cells are a dark purplish color; Gram negative cells are red to pink.

Ryu's method. The Gram reaction can also be accurately characterized by mixing a drop of concentrated bacterial suspension with a drop of 3% potassium hydroxide (KOH). Compared to the standard Gram staining procedure, Ryu's test (Suslow *et al.* 1982) is quick, simple and accurate. However, the method is not reliable for *Bacillus megaterium* and some Gram positive strains of *Clavibacter*.

To perform the test, bacteria are aseptically removed from the agar medium with a toothpick, placed on a glass slide into a drop of 3% KOH, and stirred for 10 sec using a quick circular motion. If the bacteria are Gram negative, the suspension becomes viscous and forms thread-like slime when picked up with a toothpick or a pin (**Figure 1.8**). Gram positive bacteria disperse into the drop and do not show this reaction (Suslow *et al.* 1982) because their walls do not dissolve in potassium hydroxide solution. All wheat pathogenic bacteria are Gram negative, except for species of the genus *Clavibacter*.

Figure 1.8. In Ryu's test, Gram negative bacteria are recognized by the formation of thread-like slime after stirring for 10 sec in a drop of 3% KOH.

Heat test for spores

When staining is intense, the classic Gram test may indicate whether the strain produces endospores, a characteristic of *Bacillus* spp., which are non-pathogenic on wheat. However, although *Bacillus* species are Gram positive when young, some may appear Gram negative if older cultures are used and spores are overlooked. To determine whether a bacterium produces spores, it is safer to confirm its viability after heating it. Non-pathogenic *Bacillus* strains are commonly found on plants, and the heat test helps discard these saprophytes.

For the heat test, prepare a cloudy suspension in a sterile capped tube containing 5 ml water using a fresh culture of the bacterial strain grown on nutrient agar. With a marker draw a line across the bottom of a Petri dish containing nutrient agar to divide the medium into two fields. Take a loopful of the suspension from the capped tube and streak it on one half of the agar. Incubate the capped tube for 15 min in a water bath at 80°C, making sure there is no contamination. Streak a loopful of suspension on the second field, and incubate at 27°C for 48 h. If there is growth on both halves of the agar plate, this indicates that the bacterium is spore-forming and, therefore, non-pathogenic on wheat (**Figure 1.9**). If there is no growth on the second field after 48 h, the culture is not a *Bacillus* species and should be studied further because it may be a wheat pathogen.

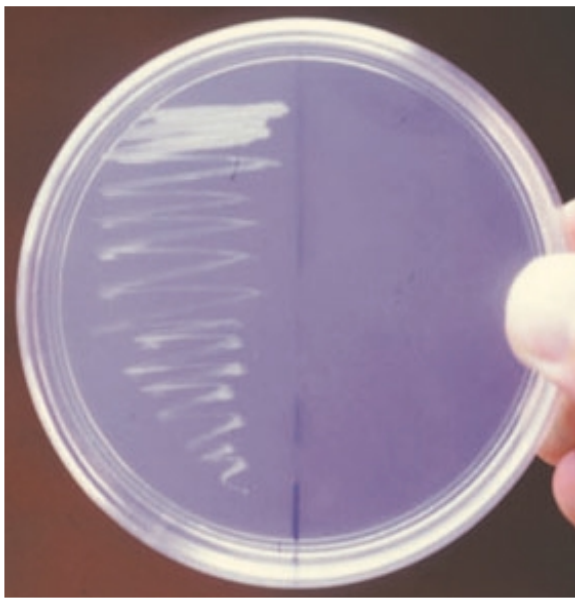


Figure 1.9. Heat test for spores: growth on the agar plate is limited to the area streaked with inoculum containing non heat-treated bacteria.

Fluorescent pigment

The production of fluorescein, a diffusible pigment, is characteristic of an important group within the genus *Pseudomonas*. A visible amount of fluorescein is produced by these bacterial species on King's medium B (see Appendix). *Pseudomonas syringae*, *P. cichorii* and *P. fuscovaginae* belong to

the fluorescent pseudomonads group, as does *P. fluorescens*, a saprophytic species frequently associated with plants.

Kovacs' oxidase

Young cultures (24 h old) grown on nutrient agar or King's medium B are necessary for this test (Kovacs 1956; Bradbury 1970). Put a drop of freshly made 1% aqueous tetra-methyl-*p*-phenylenediamine dihydrochloride onto filter paper. A small visible sample of the bacterial colony is removed with a nichrome or platinum loop and smeared onto the moistened filter. If the reaction is positive, a dark blue color (Würster blue) will appear immediately or in less than 10 sec **(Figure 10 a and b)**. The reaction is negative when no color appears or if it appears after 1 min. Some bacteria (e.g., many *Xanthomonas*) induce color in 15-60 sec; strictly speaking, they are negative but are sometimes considered to be 'delayed positive.'

The solution is toxic and carcinogenic, and should not come into contact with the skin. It should be prepared just before use and protected from light (e.g., by wrapping aluminum foil around the flask) to keep it from turning blue. The dimethyl analog of the dye can also be used, but it yields a red reaction (Sands 1990). This test is important to determine whether the bacterium has cytochrome oxidase but can be confusing if a reaction occurs after the first 10 sec and is mistakenly considered positive.

Many plant saprophytic bacteria that fluoresce on King's medium B are oxidase positive. Similarly, two wheat pathogens, *P. fuscovaginae* and *P. cichorii*, fluoresce on King's medium B and are oxidase positive; however, these species can be distinguished from non-pathogenic ones by tests such as arginine dihydrolase detection or the use of specific carbohydrates. Bacteria isolated from wheat that fluoresce on King's medium B and are oxidase negative belong to *P. syringae*.

Acid production from a carbohydrate

Among the methods proposed for detecting acid production from a carbohydrate, the following has proved to be very reliable (adapted from Dye 1962; Bradbury 1970). The recipe described below is for 1 liter (125 tubes) of medium but can be reduced depending on the amount

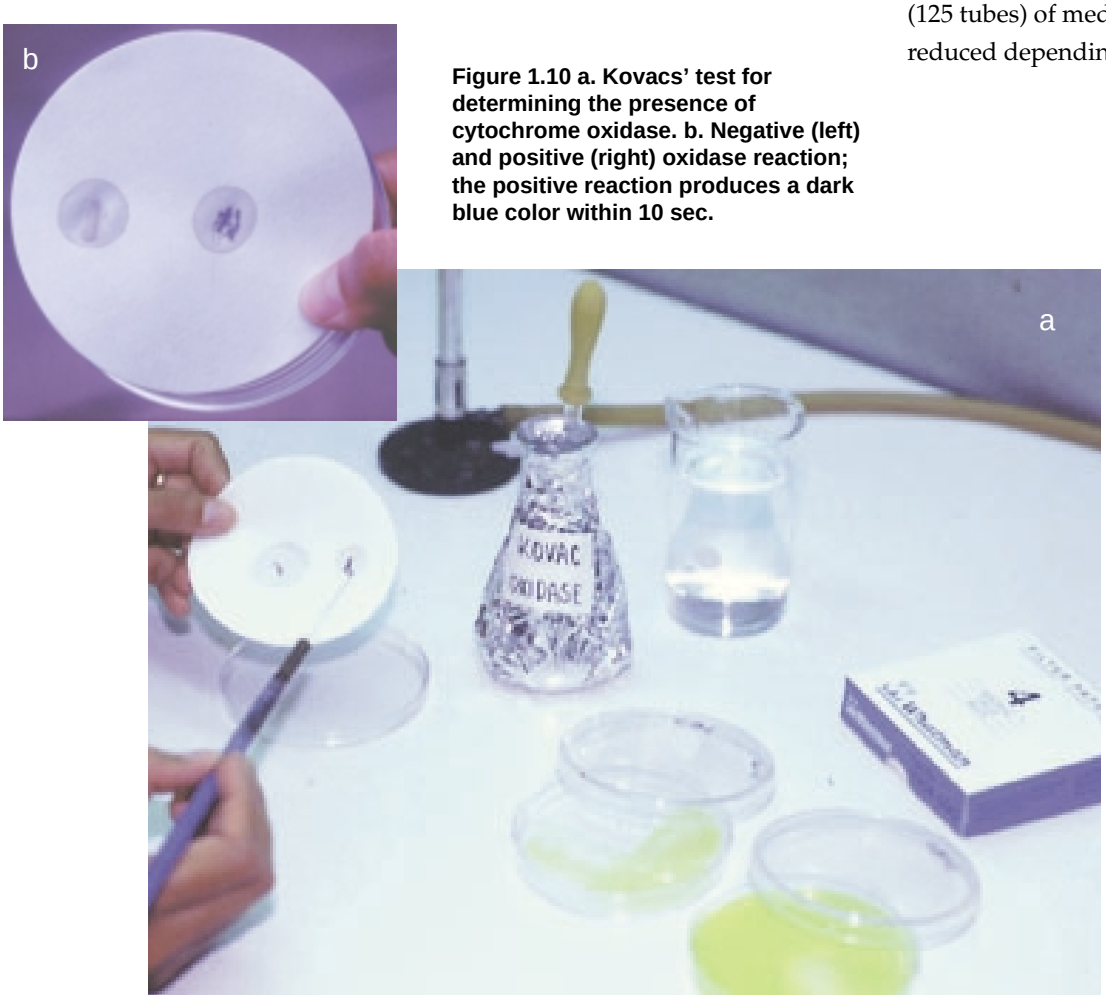


Figure 1.10 a. Kovacs' test for determining the presence of cytochrome oxidase. b. Negative (left) and positive (right) oxidase reaction; the positive reaction produces a dark blue color within 10 sec.

required. The carbohydrate source (e.g., glucose) is added at 10 g/L (1% w/v) to a basal medium prepared from the following ingredients:

$NH_4H_2PO_4$	0.5 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7\ H_2O$	0.2 g
NaCl	5.0 g
Yeast extract	1.0 g
Agar	12.0 g
Distilled water	1.0 L

Prepare the basal medium in 500 ml water and dissolve it. Add 0.7 ml of 1.5% bromothymol blue alcohol solution and adjust the pH to 7-7.2 with 40% NaOH and/or HCl. Fill test tubes with 4 ml of solution. Glucose (or the carbohydrate to be tested) (10 g) is autoclaved separately in a flask containing 500 ml water. The tubes, carbohydrate flask and a metal syringe wrapped in aluminum foil are autoclaved together for 10 min at 121ºC. After autoclaving and cooling to about 50ºC, use the syringe to aseptically place 4 ml of the carbohydrate solution in the test tubes. Tubes are vortexed before cooling.

This test is generally done with glucose as a substrate, but other carbohydrates can be used as well. Wheat pathogens can usually be identified using carbon sources such as glucose, inositol, trehalose and sorbitol. The test is particularly useful for determining whether the metabolism of a strain is oxidative or fermentative. For this reason, two tubes containing glucose are inoculated with each bacterial strain. Seal one with approximately 3 ml of mineral oil (previously sterilized by autoclaving for 10 min at 121ºC, three times) to provide anaerobic conditions. When glucose is oxidized aerobically and acid is produced, the blue medium in the unsealed tube turns yellow at the top after 48 h incubation at 27ºC (**Figure 1.11**). If the bacterium is able to use glucose under anaerobic conditions, the glucose is fermented and both tubes turn yellow. When fermentative bacteria produce gas, bubbles appear in the semi-solid medium.

This test is extremely useful for distinguishing facultatively fermentative bacteria from species characterized by an oxidative metabolism, such as all wheat pathogenic bacteria except for *E. rhapontici*.

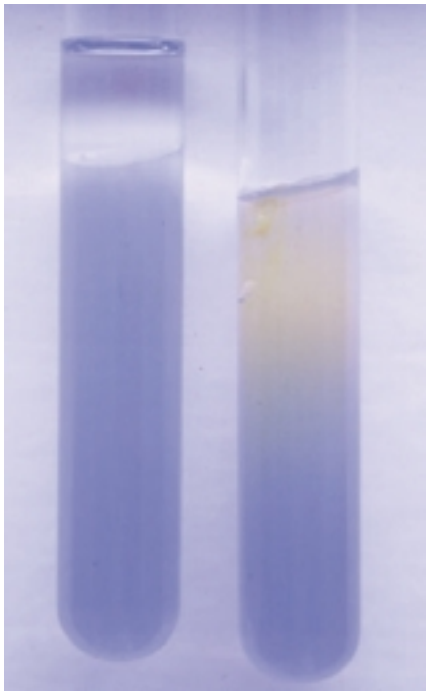


Figure 1.11. Characteristic of aerobic bacteria is acid production from glucose; after 48 h a yellow color (right) appears in the test tube that was not sealed with mineral oil.

Nitrate reduction

The nitrate reduction test (adapted from Bradbury 1970) is particularly useful for distinguishing *Xanthomonas* spp., which do not reduce nitrate, from many of the yellow saprophytes, which usually do. Among the *Pseudomonas* spp., this test comes out positive for *P. fluorescens*, a common plant saprophyte, but negative for *P. syringae* (Sands 1990).

To do this test, prepare a medium with the following ingredients:

Peptone	10 g
K_2HPO_4	5 g
Yeast extract	1 g
KNO_3	1 g
Agar	2 g
Distilled water	1 L

After dissolving the medium, pour 5 ml into test tubes and autoclave. Cool the tubes and stab inoculate with a loopful of fresh bacterial culture (24-36 h old); incubate at 27ºC for 48 h or more, until good growth is obtained. Nitrate to nitrite reduction is tested by adding the following to the culture tubes: 1) a drop of Gram's iodine (Lugol); 2) 0.5 ml of Griess reagent I (0.8% solution of sulphanilic acid in 5 N acetic acid [5 N acetic acid is obtained by mixing 57 ml glacial acetic acid and 143 ml distilled water]); and 3) 0.5 ml of Griess reagent II, which is 0.1% aqueous N-(1-naphtyl) ethylendiamine-dihydrochloride. The third step can also be accomplished by adding 5-10 drops of a solution containing 1 g of α-naphtylamine in 200 ml 5 N acetic acid. Griess reagent II must be protected from light by wrapping flask in aluminum foil.

If nitrate to nitrite reduction has taken place, the surface turns red after a few moments and the test is positive. However, if no nitrite is detected, this does not necessarily indicate that the bacterium cannot reduce nitrate to nitrite, since some bacteria produce molecular nitrogen (N_2) from nitrites, in which case nitrite is just an intermediary metabolite. To determine whether this reaction has occurred, a small

amount of zinc dust is added with a scalpel. The zinc will reduce any nitrate present to nitrite, which will then react with the reagent. After a few minutes, the surface turns red, confirming that the pathogen could not reduce nitrate to nitrite **(Figure 1.12 a, b and c)**.

Levan production

For this procedure (Lelliott *et al.* 1966; Dye and Kemp 1977), use Petri dishes containing nutrient agar (see Appendix) amended with 5% sucrose (w/v) and streak with a loopful of a pure culture of the bacterial strain. Strains that are positive for levan (a fructose polymer) production show large, mucoid, domed colonies after seven days at 25ºC.

Production of 2-ketogluconate

Production of the reducing sugar 2-ketogluconate through potassium gluconate oxidation is typical of some pseudomonads and species of *Erwinia* (Sands 1990). Since 2-ketogluconate reduces Benedict's reagent, its production can be detected by the formation of a reduced copper precipitate in the test tube when a culture several days old reacts with Benedict's reagent at 80°C (Haynes 1951; Lelliott and Stead 1987).

For this procedure Haynes (1951) placed bacteria in 300-ml Erlenmeyer flasks containing 100 ml medium and incubated them on a shaker. However, this can also be done by culturing strains in test tubes and incubating them without shaking, as follows. Fill 16-mm test tubes with 5 ml of the following medium (pH 7.0):

Tryptone	1.5 g
Yeast extract	1.0 g
K_2HPO_4	1.0 g
Potassium gluconate	40.0 g
Distilled water	1.0 L

After autoclaving tubes, inoculate them with the different strains and incubate at 28-30°C. Since tubes can be incubated for up to two weeks, cover with screw-caps to avoid contamination. After seven days,

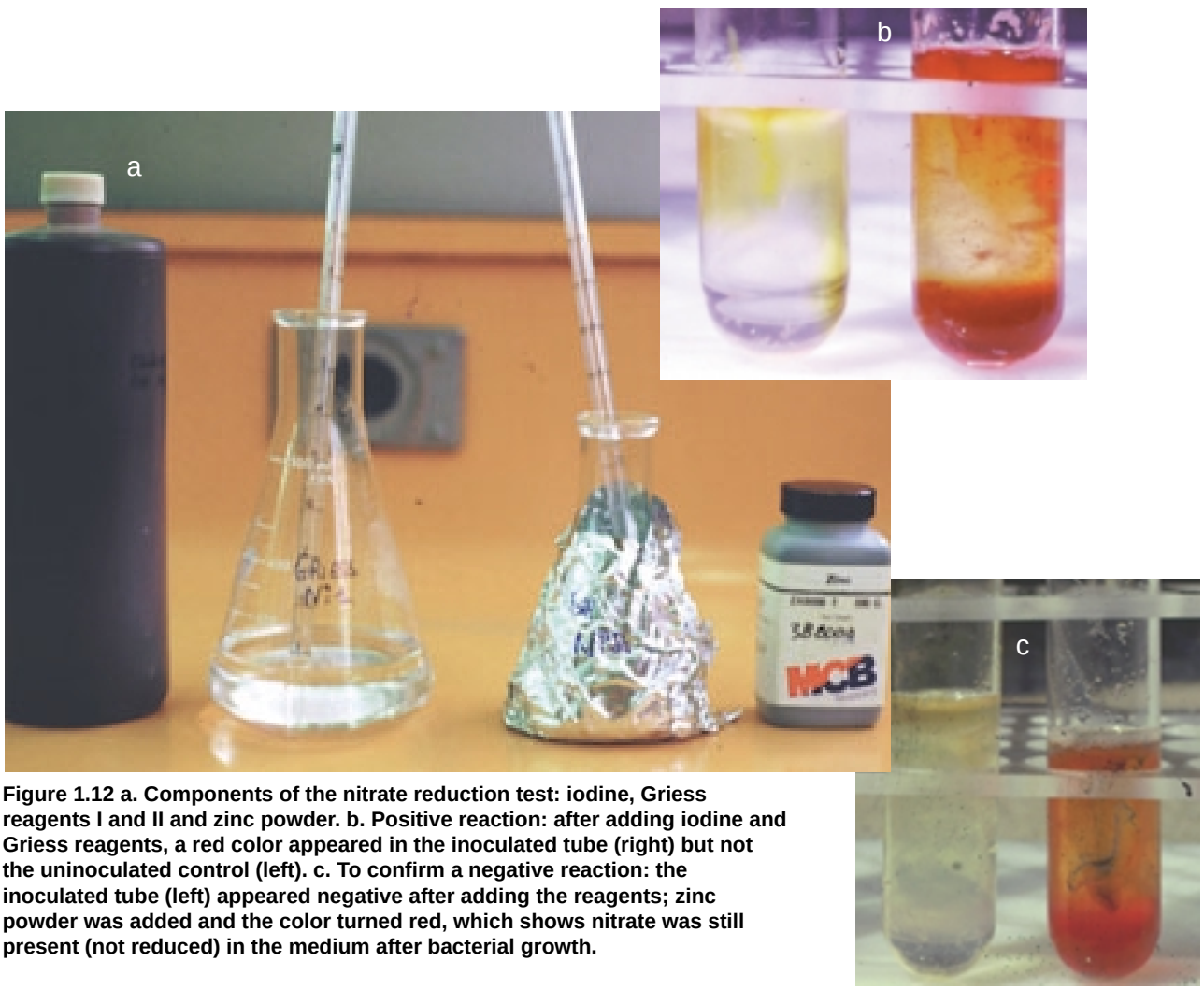
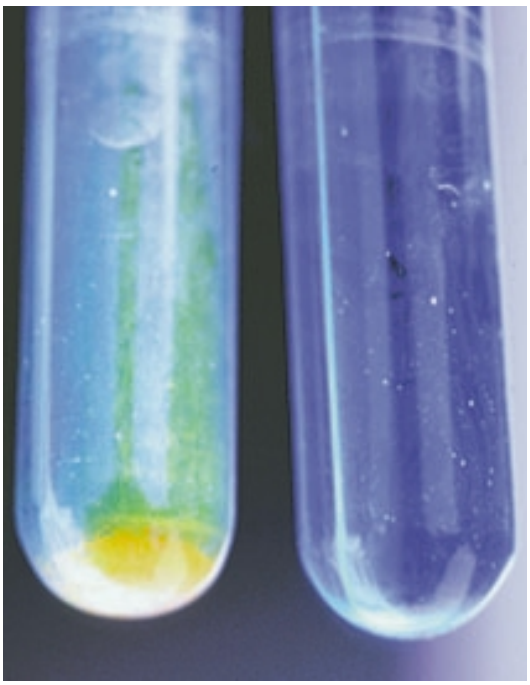


Figure 1.12 a. Components of the nitrate reduction test: iodine, Griess reagents I and II and zinc powder. b. Positive reaction: after adding iodine and Griess reagents, a red color appeared in the inoculated tube (right) but not the uninoculated control (left). c. To confirm a negative reaction: the inoculated tube (left) appeared negative after adding the reagents; zinc powder was added and the color turned red, which shows nitrate was still present (not reduced) in the medium after bacterial growth.

aseptically pipette 1 ml of the broth into an empty tube and add 10 ml Benedict's reagent. Heat tubes for 10 min in boiling water, cool rapidly in cold water and set aside until next morning. The test is positive if an orange-brown precipitate is observed (**Figure 1.13**). Haynes (1951) did this test after 2, 4, 7 and 14 days.

If not available on the market, Benedict's reagent can be readily prepared as follows (Fahy and Presley 1983). Two solutions, A and B, are prepared separately. Solution A contains 173 g sodium citrate and 100 g sodium carbonate dissolved in 600 ml hot water and diluted to 850 ml. Solution B contains 188 g copper sulfate dissolved in 100 ml water and diluted to 150 ml. Both solutions can be stored for long periods. Before using, slowly add solution B to solution A for a final ratio of 15:85. Saprophytic fluorescent pseudomonads commonly found on wheat are positive for this test, but results are negative for *P. syringae* and *P. fuscovaginae.*

Figure 1.13. Orange precipitate indicates production of 2-ketogluconate (left), compared to the control (right).



Arginine dihydrolase

Arginine, an amino acid, can be catabolized into ornithine through an enzymatic reaction that produces ATP and NH_3. The pathway is generally called "arginine dihydrolase" because it involves two enzymes that produce L-citrulline (arginine desmidase) and then ornithine (ornithine carbamoyltransferase). Certain pseudomonads are able to grow under anaerobic conditions due to this mechanism. As a result of the carbamoylphosphate (a product associated with ornithine production) reaction with ADP and carbamate kinase, ATP, CO_2 and NH_3 are produced, and the medium becomes alkaline. This can be visually observed because of phenol red, a dye that changes color depending on the pH level (**Figure 1.14**). For this test (Thornley 1960) use Thornley's medium 2A, which contains:

Distilled water	1.00 L
Peptone	1.00 g
NaCl	5.00 g
K_2HPO_4	0.30 g
Agar	3.00 g
Phenol red (dissolved in 10 ml ethanol)	0.01 g
L(+) arginine HCl	10.00 g

Mix the medium and boil to dissolve the agar, which has a pale pink color at pH 7.2. Pour 5 ml into screw-cap tubes and autoclave at 121°C for 10 min. Avoid excessive autoclaving, since it may cause arginine degradation, which in turn may induce an alkaline shift and change the color of the medium, making the determination of results difficult or unreliable.

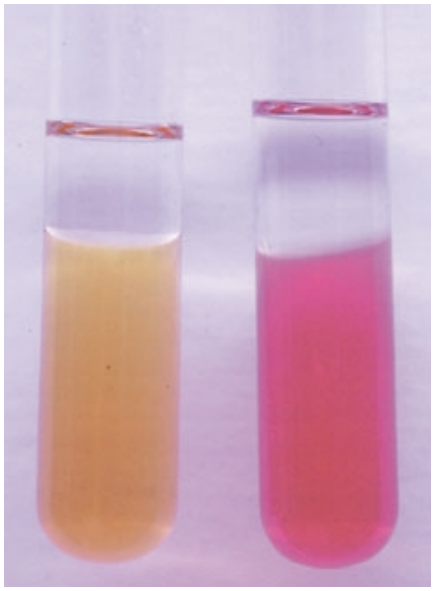


Figure 1.14. In the arginine dihydrolase test, a positive reaction is characterized by the appearance of a reddish purple color after stab inoculation (right); controls are pink (left).

Stab inoculate the tubes and cover with 1 cm paraffin oil previously autoclaved for 10 min at 121ºC, three times. Incubate for three days at 27°C. The test is positive if there is a definite change in color, to red, under anaerobic conditions. If there is no change in color, the test is negative. *Pseudomonas fluorescens* and *P. fuscovaginae* test positive, but *P. cichorii* and *P. syringae* test negative.

Potato soft rot

For this test (Bradbury 1970), well washed, firm potatoes are surface sterilized with 70% ethanol and then peeled under microbe-free conditions. Use a hole punch to cut plugs about 2 cm in diameter. With a

flamed scalpel cut plugs into slices about 0.7 cm thick. Place slices in a sterile Petri dish and pour sterile water over them to a depth of 3 mm, or place on filter paper moistened with sterile water. With a sterile needle, place a relatively large amount of inoculum in the middle of each piece and incubate for 24-48 h at 25ºC. Soft rotting bacteria such as *E. rhapontici* produce massive rot in 24 h; however, many other species, including saprophytic pseudomonads and *Bacillus* spp., also show pectolytic activity, although it is usually not very intense (Lelliott and Stead 1987). *Pseudomonas syringae* tests negative for this procedure.

Esculin hydrolysis

All *X. campestris* pathovars hydrolyze esculin through a ß-glucosidase reaction that results in glucose and dihydrocoumarin, which is dark brown in color (Sands 1990) (**Figure 1.15**). For this procedure (Lelliott and Stead 1987), mix, dissolve, and heat 10 g peptone, 1 g esculin, 0.5 g ferric ammonium citrate, and 12 g agar in 1 liter distilled water. Pour 5 ml into each test tube. Autoclave tubes at 121ºC for 15 min and stab inoculate. The reaction is positive if there is progressive darkening within 3-4 days and fluorescence disappears under UV light.

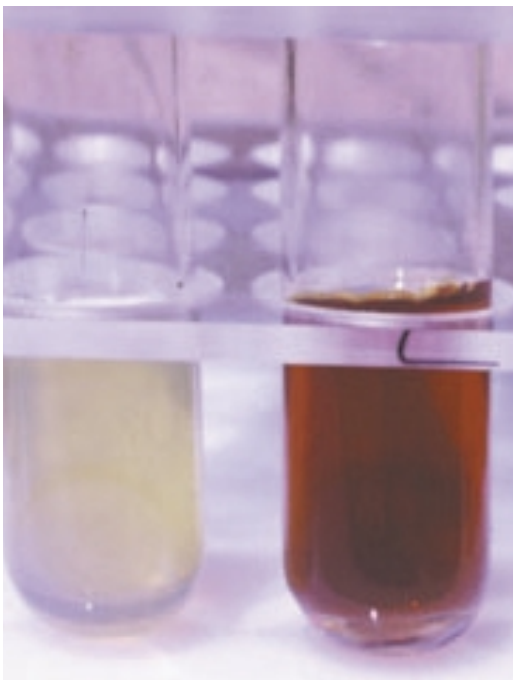


Figure 1.15. Esculin hydrolysis is characterized by the production of a dark brown color (right).

Hypersensitivity reaction

Practically all phytopathogenic bacteria that cause tissue necrosis in a susceptible host induce a hypersensitive reaction on tobacco leaves that have been inoculated with a cloudy bacterial suspension (approximately 10^8 to 10^9 cells/ml). To produce this reaction, infiltrate the suspension with a hypodermic syringe into the intercellular space of a healthy tobacco leaf. If the suspension contains a wheat pathogenic bacterium, the injected tissue turns necrotic within 24 h. This reaction may be less marked with *Xanthomonas*, for which a cool incubation temperature (15-20°C) is suitable. Non-pathogenic bacteria do not cause necrotic symptoms, though occasionally some chlorosis may develop a few days after inoculation. While this test (Klement 1990) helps to confirm that a strain is phytopathogenic, it is not a substitute for the pathogenicity test on a susceptible host. However, it may suggest that determinative tests for wheat pathogenic bacteria are needed.

Pathogen Characterization Using Serological Methods

Although serology is almost as old as plant pathology itself (Schaad 1979), in recent years it has progressed significantly with the development of monoclonal antibodies (Mabs) (Koehler and Milstein 1975) and the improvement of techniques using antisera (Clark and Adams 1977). These techniques are powerful tools for specifically identifying plant pathogenic bacteria, conducting epidemiological studies and understanding plant pathogen interactions through the detection of compounds produced by bacteria. Serological methods have been developed for identifying several wheat pathogens.

Polyclonal antibodies, which are easily obtained through animal immunization (e.g., rabbits), actually contain different antibodies that react with epitopes on bacterial cells (Lelliott and Stead 1987). Their composition varies depending on the animal that generated them. Mabs are produced by cell culture and fusion technology in which the fused cells produce a single type of antibody. In contrast with polyclonal antisera, Mabs have the advantage of being specific to a single epitope and can be selected for a particular affinity or use. Also, a new batch of the same antibody can be produced whenever needed since the cell line can be maintained

The Pathovar Concept

Before the 1940s, new bacterial species were described because they had been isolated from new hosts. This "new" host/"new" species rule resulted in a vast and indeterminate number of phytopathogenic bacterial species, especially in the *Pseudomonas* and *Xanthomonas* genera. In some cases, neither the host range nor the relatedness of new species to already described ones were studied thoroughly (Starr 1959). But even species that clearly differed in the range of host plants on which they were pathogenic were often not distinguishable using routine *in vitro* bacteriological tests (Misaghi and Grogan 1969; Sands *et al*. 1970; Doudoroff and Palleroni 1974). As a result, the Approved Lists of Bacterial Names of the International Committee on Systematic Bacteriology (Skerman *et al*. 1980) discarded many commonly accepted names of phytopathogenic bacteria. According to these lists, bacteria included in group I of Lelliott *et al*. (1966) constitute one species, *P. syringae*. Similarly, many phytopathogenic xanthomonads were brought together under the name *X. campestris*.

The term pathovar (originally recommended by Ribeiro *et al*. 1977) was introduced to conserve the former specific names for future use by plant pathologists. According to Young *et al*. (1978), plant pathogenic bacteria that do not have a claim to species rank in the approved lists should be classified as pathovars. The term pathovar was defined as "a strain or set of strains with the same or similar characteristics, differentiated at the infrasubspecific level from other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more plant hosts" (Dye *et al*. 1980).

In the future, more sophisticated taxonomical methods may reveal stable and reproducible differences between pathovars, justifying the reinstatement of former species. The term *forma specialis* is defined as a subdivision of a species of parasitic or symbiotic microorganisms distinguished primarily by adaptation to a particular host (Anonymous 1966). The term pathovar thus appears to be better suited for bacterial pathogens, a number of which have a wide host range. *Pseudomonas syringae* pv. *syringae*, in particular, is an extremely heterogeneous pathovar with a very broad host range that, in addition to wheat, includes lilac, stone fruit trees, bush bean, and many weeds. This atypical feature of pv. *syringae*, in contrast to most other pathovars characterized by a rather narrow host range, is due to the common practice of using pv. *syringae* as a repository for all those pathovars of *P. syringae* whose exact host range is unknown (Rudolph 1995). *Pseudomonas syringae* pv. *atrofaciens*, on the other hand, is a very homogeneous group of strains that almost exclusively infects cereals and causes the typical basal glume rot (von Kietzell 1995). It is thus not surprising that within the heterogeneous *Pseudomonas syringae* pv. *syringae* a few strains that are very similar to pv. *atrofaciens* have been found (von Kietzell 1995). Nonetheless, the distinct homogeneity of pv. *atrofaciens* strains as compared to pv. *syringae* strains and their induction of very specific disease symptoms on wheat and barley justified differentiating pv. *atrofaciens* from the large variable group of pv. *syringae* strains.

Wide host range variation among single strains has been reported in the "*X. translucens*" group. Also, the pathovar names currently in use can be confusing because strains having different names may be similar or because host range was not clearly defined after the pathogen was isolated. However, RFLP patterns and pathogenicity studies confirm that there are two distinct groups of strains: strains pathogenic on wheat and barley (*X. t.* pv. *undulosa*) and strains pathogenic on barley but not on wheat (*X. t.* pv. *translucens*) (Bragard *et al*. 1995). A list of *P. syringae* and *X. campestris* pathovars was published by Dye *et al*. (1980) and completed by Bradbury (1986), Hayward (1993) and Young *et al*. (1996).

for years by cryopreservation. However, compared to polyclonal antisera, Mabs are about ten times more expensive, may have lower affinity and sensitivity, and may be less useful for techniques involving precipitation.

Both polyclonal and monoclonal antibodies can be used in several serological applications: agglutination, immunodiffusion assay, immunofluorescence microscopy (Epi-UV), enzyme-linked-immunosorbent assay (ELISA) and dot-blot immunoassay (Dot-Elisa or Biodot). It can also be used to produce so-called monospecific antibodies (MSA) (Baharuddin *et al.* 1994). Compared to ELISA or immunofluorescence, the immunocolony blot test with MSA has two advantages: it does not require expensive laboratory equipment, and MSA can be produced at a lower cost than monoclonal antibodies. Though highly specific, MSA are not as sensitive as conventional polyclonal antisera. For more details on serological techniques, see Hampton *et al.* (1990) and Schaad *et al.* (1990).

Agglutination

When a compatible reaction occurs between bacterial cells and a specific antiserum, a precipitate forms and causes agglutination. To observe this process, mix 30 µl of a bacterial cell suspension (10^9 cfu/ml in 0.85% saline solution) and 30 µl of the antiserum (pure or diluted) on a clean microscope slide. The reaction should be observed within a couple of minutes to half an hour under a dark field microscope. If a dark field microscope is not available, agglutination can be detected by observing the slide with the unaided eye under indirect light and over a dark background. The technique is simple, fast, and easily performed with limited facilities. It has been applied for characterizing xanthomonads on small grains (Elrod and Braun 1947a) and, more recently, for identifying *P. fuscovaginae* on wheat seed (Duveiller and Martinez 1990).

The limitations of the technique are antiserum specificity and the need to work with a pure bacterial culture. Also, a large quantity of antiserum is used, and the reaction depends on the antigen:antibody ratio. A preliminary test should therefore be performed using different antibody and bacterial concentrations to determine the optimum ratio for observing the reaction.

Ouchterlony double immunodiffusion

In this method, antibodies and antigens are deposited in different wells punched into buffered agarose. Then antibodies and antigens migrate through the agarose toward each other, forming a precipitin line where they meet. Although the sensitivity of this technique is low, it does indicate the relationship between different antigens. Its main disadvantages are the need to use only pure bacterial cultures (antigens), a long incubation period (three days or more) and the need to determine the optimum antigen:antibody ratio. The latter can be achieved using Piazzi's test (Piazzi 1959). The method is considered useful for identifying small numbers of strains (Stead 1992).

The use of bacterial extracts, as well as the filtration effect through the agarose, artificially increases specificity compared with more sensitive techniques such as immunofluorescence. This has been demonstrated by several authors who used polyclonal antisera in the identification of xanthomonads pathogenic to small grains (Azad and Schaad 1988b; Bragard and Verhoyen 1993).

Immunofluorescence (IF)

Bacterial cells are fixed onto a microscope slide using heat or ethanol and labeled with a specific antibody. In the direct immunofluorescence method, the bacterium reacts to an antibody attached to a fluorochrome (usually fluorescein or rhodamine). The fluorochrome is observed under epi-UV light using a UV microscope and immersion oil; fluorescing cells appear green on a dark background.

The indirect IF method is often preferred over direct IF because it is more sensitive and more specific. In indirect IF, the bacterium reacts against a first antibody, which in turn reacts with a second fluorochrome-labeled standard antibody (**Figure 1.16**). This avoids the more difficult labeling of the direct technique. Sensitivity is generally increased because several antibodies can be attached to the one that recognizes the pathogen.

The highly sensitive indirect IF technique has a detection limit of 10^3–10^4 cells/ml. Since cell morphology can be verified, false positive reactions due to cross-reactions may be reduced. It is also possible to count reacting cells. The main limitations of the method are the specificity of the antiserum, the small volume of sample observed when IF is used in quantitative detection of the pathogen, and the high cost of an epifluorescence microscope.

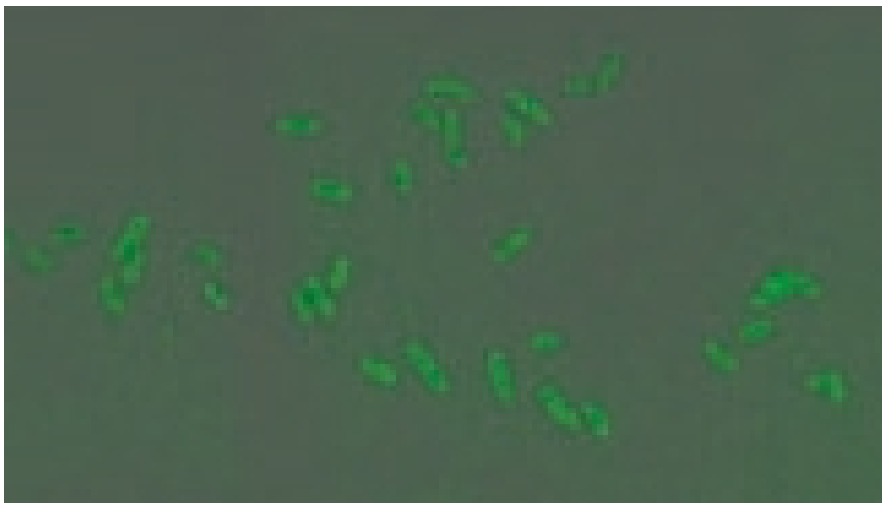


Figure 1.16. *Xanthomonas translucens* pv. *undulosa* cells observed under immunofluorescence microscopy.

Bragard and Verhoyen (1993) produced Mabs for the detection of xanthomonads from small grains using IF. Western blot analysis indicates the reaction of such antibodies with the lipopoly-saccharide O-side chain of the bacterium, which makes it possible to detect the pathogen by enzyme-linked immunosorbent assay (ELISA) (Bragard, unpublished). If IF is used in the detection of pathogenic xanthomonads from wheat seed, it should be combined with another method, such as dilution plating on a semi-selective medium, to increase the accuracy of the results (Duveiller and Bragard 1992).

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a sensitive assay combining the specificity and affinity of antibodies with the enzymatic reaction of a chromogenic substrate. Several protocols describing sandwich, direct, and indirect procedures have been published. Each of them was developed for special working conditions. In general, the antigen (bacterial cells, cell extracts, antigens released in liquid medium, etc.) is fixed directly onto a 96-well microtitration plate, an operation sometimes referred to as immunotrapping. The spaces not covered by the antigen are blocked with bovine serum albumin (BSA), skim milk or gelatin. Reacting cells are detected by an antibody attached to an enzyme (alkaline phosphatase or horseradish peroxidase) that induces color after enzymatic reaction with its substrate (e.g., nitrophenyl-phosphate or phenilene-diamine). The reaction is evaluated using an automated photometer that compares the color in each well with appropriate controls on the plate, but results can also be assessed by the unaided eye.

Since each well in the 96-well plate contains a sample, ELISA allows large numbers of samples to be tested per day. However, the method has several drawbacks that hinder its wide application in the identification of plant pathogenic bacteria. The detection threshold is rather high (10^4-10^5 cells/ml) compared to IF. Also, although a poly-l-lysine fixation procedure was developed to limit this effect, fixing bacterial cells onto the plate generally presents problems, and bacteria are easily detached during washing procedures. In addition, exogenous bacterial activity has to be taken into account and monitored using appropriate controls. All xanthomonads present alkaline phosphatase activity, which reduces the interest of this enzyme for ELISA (Bragard and Verhoyen 1993). Finally, false positives may occur when analyzing dusty wheat seed samples due to a possible confounding effect during the absorbance measurement of the enzymatic reaction.

Frommel and Pazos (1994) proposed using a polyclonal antiserum for *X. t.* pv. *undulosa* detection in ELISA. Bragard *et al.* (1993) evaluated ELISA for detecting *X. t.* pv. *undulosa* with Mabs in wheat seed and compared the method to other seed indexing techniques such as the seedling injection technique (Mehta 1990), dot-immunobinding and immunofluorescence (Duveiller and Bragard 1992).

Dot-immunobinding assay

Dot-immunobinding assay (Lazarovits 1990), also known as DIA or DIBA, is a variation of ELISA modified in two ways. First, the antigen is trapped on a nylon or nitrocellulose membrane, not on the microtitration plate; this can be done with a blot-dot microfiltration apparatus. Second, the enzymatic reaction peroxidase with perhydrol in the presence of the chromogenic substrate (e.g., 4-chloro-1-naphtol) produces a precipitate that appears as colored dots on the membrane. Though faster and easier than ELISA, the technique has similar drawbacks:

low sensitivity compared with IF, fixation of bacteria, exogenous bacterial activity, and false positive reactions when dusty seeds are tested. However, it has been used successfully by Claflin and Ramundo (1987) to detect "*X. translucens*" in seed using polyclonal antibodies, and by Duveiller and Bragard (1992) using a Mab.

Pathogen Characterization Using Molecular Methods

Recent developments in biotechnology have provided powerful, new molecular tools for quick, precise identification of bacterial pathogens, sometimes at the strain level. These techniques are more sensitive than conventional methods and often more suitable for detecting bacteria on symptomless leaves or in seed samples. Though not yet widely available, these techniques may rapidly find new applications. This section presents a brief overview of the range, uses and limitations of several molecular methods based on nucleic acid hybridization. In contrast with techniques based on phenotypic features (such as serology), they are related to genomic traits, which increases specificity.

Plasmid and genomic fingerprinting

Plasmid or genomic DNA is digested by restriction endonucleases, which are enzymes that cut DNA at a specific site. The resulting fragments are separated on agarose or acrylamide gels and stained with either ethidium bromide or silver (**Figure 1.17**). The number of fragments is very high, which makes it difficult to compare genomic fingerprints without the aid of a computer. Lazo and Gabriel (1987) used this technique for comparing strains and pathovars. Care should be taken when choosing which restriction endonuclease and experimental conditions to use; if not appropriate, incomplete digestion could produce misleading patterns.

Restriction fragment length polymorphisms (RFLPs)

Digested DNA separated on agarose gels can be transferred as single-strand DNA fragments onto nylon or nitrocellulose membranes using a Southern blot. These fragments are hybridized with a complementary single-strand DNA or RNA probe, labeled with radioactive ^{32}P (which is detected by autoradiography) (**Figure 1.18**), or with such chemicals as

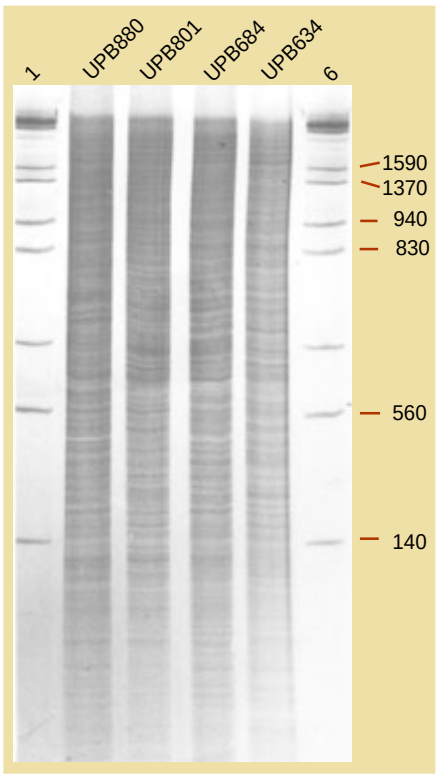


Figure 1.17. Genomic fingerprinting: genomic DNA of *Xanthomonas translucens* strains digested with *Pst*YI restriction endonuclease; separation on 7% SDS-PAGE and silver staining. Lanes 1 and 6 correspond to molecular mass standard (Lambda phase DNA, *Eco*RI and *Hind* III digested); UPB880, UPB801 and UPB684 are *X. t.* pv. *translucens* strains isolated from barley in Libya, Paraguay and Iran, respectively; strain UPB634 is a *X. t.* pv. *undulosa* isolated from barley in Mexico. Reproduced by permission of C. Bragard.

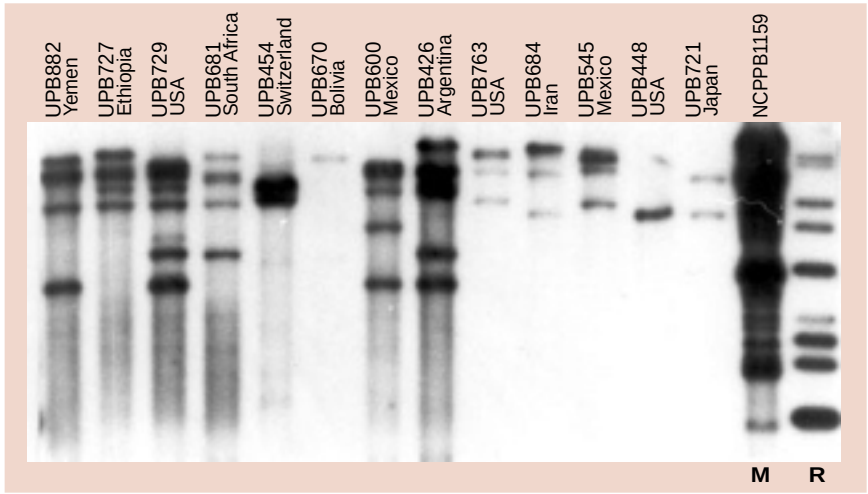


Figure 1.18. Southern hybridization of *Eco*RI digested genomic DNA of 13 *Xanthomonas translucens* strains from several countries pathogenic to small grains using plasmid probe pBSF2 from *X. c.* pv. *manihotis.* M = *X. c.* pv. *manihotis* NCPPB1159; R = molecular mass standard Raoul I (Appligene). Reproduced by permission of C. Bragard.

acetylaminofluorene or digoxygenin, detected by an immuno-enzymatic reaction.

Although time-consuming and not appropriate for detecting pathogens in plant samples, the technique has proved very useful in identifying pure cultures of plant pathogenic bacteria at the pathovar and even the strain level. It has also been used with pathogenicity-related probes for studying variations among xanthomonads that are pathogenic to small grains (Alizadeh *et al.* 1997; Bragard *et al.* 1995).

Macro-restriction analysis of DNA molecules by electrophoresis

Recently developed techniques allow analysis of large DNA molecules by gel electrophoresis. DNA molecules of up to 10,000 Kb can be characterized using pulsed field gel electrophoresis (PFGE) (Schwartz and Cantor 1984), which is based on changes in molecular structure that occur as molecules migrate through an alternately oriented field in agarose gel. In field inversion gel electrophoresis (FIGE), pulsing is effected by periodic inversion of the field, allowing multiple samples to be run collinearly. Several pathovars of *P. syringae*, including *syringae* and *atrofaciens*, were differentiated from each other with this technique (Grothues and Rudolph 1991).

Dot-blot and colony blots

A quick alternative to RFLPs, a dot-blot apparatus is used to bind extracted genomic DNA in spots on a membrane. Spots containing DNA are then subjected to hybridization with a specific probe, as for RFLPs.

In colony blotting, a nylon membrane is applied directly onto colonies growing on an agar plate under alkaline conditions to allow cell lysis and DNA denaturation. The membrane is then used for hybridization, as it is in the dot-blot procedure. The dot-blot technique using probes derived from a *X. t.* pv. *undulosa* strain has been used successfully to distinguish strains of the "*X. c. translucens*" group from other *Xanthomonas* pathovars. It should be noted that in the dot-blot technique the level and purity of DNA (less controlled in colony blots), as well as the influence of stringency conditions during washing, are extremely important.

Polymerase chain reaction (PCR)

Polymerase chain reaction can be defined as *in vitro* enzymatic synthesis of specific DNA sequences generally using two primers (oligonucleotides) that hybridize with complementary DNA strands. The reaction involves: 1) denaturation at high temperature (about 94ºC) to separate the double strands of DNA, 2) annealing at low temperature to attach specific primers to the single DNA complementary sequence, and 3) an elongation step at intermediate temperature (72ºC) for polymerizing targeted DNA fragments. This cycle is repeated 30-45 times, resulting in the exponential accumulation of the desired DNA fragment(s). Amplified fragment(s) are shown by acrylamide or agarose electrophoresis and silver or ethidium bromide staining. The technique can be combined with ELISA (ELISA-PCR) to provide potential new applications, particularly when high sensitivity is required.

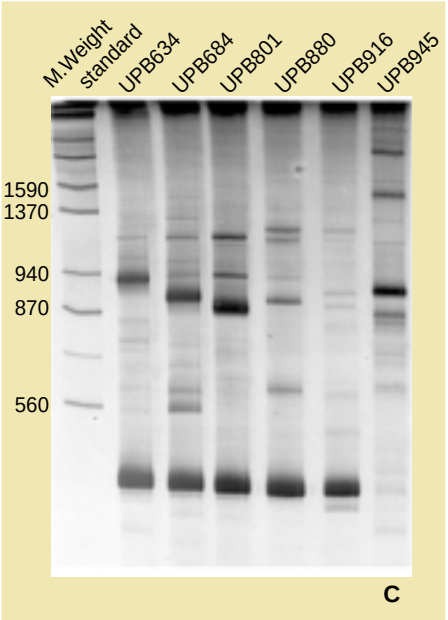


Figure 1.19. REP-PCR electrophoresis of genomic DNA of *Xanthomonas translucens* pv. *translucens* strains compared with a *X. t.* pv. *cerealis* strain (C); separation on 12% SDS-PAGE and silver staining. Strain UPB634 is a *X. t.* pv. *undulosa* isolated from barley in Mexico; strains UPB684, UPB801, UPB880 and UPB916 are *X. t.* pv. *translucens* strains isolated from barley in Iran, Paraguay, Libya and the USA, respectively; strain UPB945 is a *X. t.* pv. *cerealis* isolated from *Bromus inermis* in the USA. Reproduced from Bragard 1996, by permission of the author.

PCR has been proposed for detecting "*X. translucens*" using specific primers with a detection threshold of 10^3 cells/ml (Maes and Garbeva 1994). Also, REP-PCR was proposed by Louws *et al.* (1994) for the identification of xanthomonads and has been applied by Bouchgua (1995) and Bragard (1996) (**Figure 1.19**). The major limitations of this technique are its high cost and that the amplification reaction may be

inhibited when plant or soil extracts are tested, probably due to inhibition of polymerase activity or to a non-specific reaction with non-target DNA.

Strain Conservation

It is often necessary to maintain bacteria in viable, pathogenic condition for future research and diagnostic purposes. Several methods have been devised to preserve these pathogens without contamination for long periods of time.

The best method for long-term preservation of bacteria is lyophilization (Lelliott and Stead 1987; Stead 1990). Many international culture collections have adopted this method because it keeps bacteria alive for 10 years or more. The procedure is as follows.

The bacteria are grown on King's medium B (if available) so as to have them ready for processing in two or three days. Prepare two solutions: one 14% sucrose and another 14% beef extract peptone. Sterilize them separately and then mix them together in equal parts. Make a bacterial suspension in 20 ml of the resulting sterile lyophilization medium. Centrifuge this dense suspension (for 10 min at 10,000 g) in 4 ml of the sucrose-peptone medium. Discard the supernatant and suspend the bacteria again in 4 ml of the same sucrose-peptone medium. Aseptically pour 0.2 ml of the suspension into each 1-2 ml ampoule without touching the sides. Place a cotton plug on top of each ampoule and push it down to about 3 cm from the base. Lyophilize in a freeze dryer for 18-24 h. Use a propane flame to seal the ampoules and store at 4°C. To revive the bacteria, cut the ampoules and add 0.1 ml of a 0.1% sterile peptone solution. With a sterile triangle rod, streak the suspension on a fresh agar plate. Incubate up to 10 days at 27°C.

When a freeze dryer is not available, pure cultures can be grown on GYCA slants (see Appendix) for 4-5 days and kept at 4°C for several months (**Figure 1.20**). The medium contains $CaCO_3$, which reduces acidification. "*Xanthomonas translucens*," *P. syringae* and *P. fuscovaginae* can easily be kept for several months to a year under these conditions. A similar form of preserving bacteria is to grow them in neutral slant medium for 24-48 h. One centimeter of mineral oil is added to cover the slant and eliminate desiccation. The tubes are maintained at 4°C or at room temperature.

There are several other methods for preserving plant pathogenic bacteria. For example, bacteria in high concentrations (10^9 cell/ml or more) can be suspended in sterile distilled water and kept in screw cap bottles at 18-22°C. Another method entails preserving bacteria either in sterile soil at pH 7 or in soil that has buffering capacity at 18-22°C. Bacteria, not necessarily in pure state, can also be maintained in diseased tissue at -20 to -70°C. Yet another option is to inoculate bacteria onto porcelain beads containing silica gel or onto gelatin discs supplemented with ascorbic acid and dried with the help of phosphorus pentoxide. Finally, bacteria can be preserved by periodically cultivating them on different media, but it must be noted that their life span may be only 1-10 weeks and that their pathogenicity or aggressiveness may change after several transfers.

Figure 1.20. Yellow slime accumulates at the bottom of GYCA slants used to conserve *Xanthomonas translucens* strains; tubes can be kept for several months at 4°C.

Chapter 2 : Bacterial Leaf Streak and Black Chaff Caused by *Xanthomonas translucens*

E. Duveiller, C. Bragard and H. Maraite

Bacterial leaf streak (BLS) of cereals, also known as bacterial leaf stripe, is caused by *Xanthomonas translucens* (ex Jones, Johnson and Reddy 1917) Vauterin, Hoste, Kersters and Swings 1995. The disease, called black chaff when on the glumes, is seed-borne and a constraint for international germplasm exchange. Black chaff has been reported since the end of last century; however, some reports are misleading because disease symptoms on the ear are often confused with the abiotic stress known as pseudo-black chaff or brown melanosis (Broadfoot and Robertson 1933; Hagborg 1936).

The pathogen was first identified on barley (*Hordeum vulgare* L.) (Jones *et al.* 1917), and later on wheat (*Triticum aestivum* L.) (Smith *et al.* 1919), rye (*Secale cereale* L.) (Reddy *et al.* 1924), grasses (Wallin 1946a) and finally on triticale (X *Triticosecale* Wittmack) (Zillinsky and Borlaug 1971). Different names have been proposed, depending on the host plant, but the name *X. campestris* pv. *translucens*, first assigned to the barley pathogen originally described by Jones *et al.* (1917), has been used for any cereal streak pathogen even when the host range was undetermined (Bradbury 1986). As a result, data on host specialization, on the association (or non-association) of a pathogenic bacterium with black glumes (particularly in early reports), and on the importance of the disease have been confusing. BLS is usually considered to be widespread but unimportant (Lelliott 1987). However, accounts of this disease, particularly on wheat in the 1980s, have become more frequent and have aroused much concern (Duveiller 1989).

Names currently included in the International Society of Plant Pathology's (ISPP) list of plant pathogenic bacteria (Young *et al.* 1996) are used in this manual. Hence, the name *X. t.* pv. *undulosa* is used here to refer to the pathogen that causes BLS on wheat, except when the authors themselves have used another name. Since pathovar names are not used consistently by all authors, in this manual we use the designation "*X. translucens*" in a broad sense when it is not known which pathovar is involved or to refer to the group of pathovars that cause BLS on cereals and grasses.

Distribution

Most *Xanthomonas* strains causing BLS symptoms have been isolated from samples collected in experiment stations and not in commercial fields, where they are less commonly found. This may be because scientists who are familiar with bacterial problems make more intense observations in experiment stations. Whatever the reason, BLS is found mainly in breeding stations, where the problem has been ignored for years (Schaad 1987a).

Bacterial leaf streak occurs over a range of very different conditions such as sprinkler irrigated fields in temperate climates, high rainfall subtropical highlands, and warmer environments characterized by cool nights or frequent climatic changes and sudden temperature variations. Although reports are generally sporadic, they come mostly from warmer cereal-growing areas.

In North America, BLS has been reported in several states of the USA (Heald 1906; Jones *et al.* 1916; Smith 1917; Johnston 1929; Melchers 1930; Milus and Kirkpatrick 1990; Murray and Malloy 1990). Outbreaks have not been observed in Canada recently (Hagborg 1934; 1968; 1974). In Mexico, the disease was observed in the northeast in 1931 (Bamberg 1936) and is most severe today in areas of high elevation such as the temperate and humid central highlands of Toluca (2,650 m above sea level) (Duveiller 1989). It is often found in experiment stations due to the broad range of genotypes sown, some of which are quite susceptible. In South America, wheat is the most affected crop, although BLS is also found on other small grains and grasses (Mehta 1990; Duveiller *et al.* 1991). The disease occurs in Argentina, Bolivia, parts of Brazil, Paraguay, Peru and Uruguay (Mehta 1990; Duveiller,

1989; Pereira *et al.* 1983; Luzzardi *et al.* 1983; Mohan and Mehta 1985; Duveiller *et al.* 1991; Frommel 1986; Tessi 1949; Abadie 1988).

In Asia, the disease is known on wheat in China (Chen and Ding 1981; Sun and He 1986), Pakistan (Akhtar and Aslam 1985 and 1986) and Iran (Alizadeh and Rahimian 1989; Alizadeh *et al.* 1995), and on triticale in India (Richardson and Waller 1974). In the Near and Middle East, it affects durum (*Triticum turgidum* var. *durum* L.) and bread wheat in irrigated areas of Syria (Mamluk *et al.* 1990), Israel (CIMMYT 1977), Turkey (Sands and Fourest 1989; Demir and Üstün 1992), and Yemen (Maraite and Ferauge, personal communication). Gorlenko (1960) noted the occurrence of *X. t.* pv. *translucens* and *X. t.* pv. *secalis* in the Omsk region (Russia) and BLS was also reported in Krasnoyarsk (Dobretsov 1963) and the Novosibirsk area (Bushkova 1966). It has also been found in Malaysia (Sabah, as cited in Bradbury 1986). In Japan, *X. t.* pv. *translucens* and *X. t.* pv. *cerealis* have been found on several gramineae but not on wheat (Tominaga 1967; Miyajima 1980 and 1982; Miyajima and Tsuboki 1980).

In Europe, the first reports of black chaff came from France, Belgium and Russia (Millasseau 1928; Hocquette 1929; Marchal 1930 and 1932, Gorlenko *et al.* 1939). However, Marchal (1948) later denied that black chaff was present in Belgium. The disease currently seems to be absent from western Europe (Paul and Smith 1989), probably due to unfavorable environmental conditions, particularly low temperatures. Nonetheless, sporadic outbreaks on barley in Spain (Lopez *et al.* 1987; Noval 1989) and on triticale (Wolski, personal communication) and wheat (Arseniuk, personal communication) in Poland indicate that the risk in western Europe should not be underestimated.

In Africa, BLS has been found in Kenya (Burton 1930), Ethiopia (Korobko *et al.* 1985), South Africa (Vervoerd 1930; Smit and Van A. Bredenkamp 1988), Tanzania (Bradbury 1986), Libya and Madagascar (Bragard *et al.* 1995), and Morocco (Sands and Fourest 1989).

In Australia (Moffett 1983), BLS has been recorded on wheat and rye in New South Wales (Noble *et al.* 1934; Noble 1935), and *X. t.* pv. *cerealis* was identified on Japanese millet (*Echinochloa crus-galli* var. *frumentacea* L. Beauv.) (Moffett and McCarthy 1973) in Queensland.

Importance

Little quantitative information is available on losses caused by BLS. Measuring yield losses in commercial fields is not easy because the lack of an effective treatment makes it impossible to grow healthy control plots. Moreover, when favorable conditions are present, the disease may develop very fast within a region, making it difficult to observe different infection levels in a range of plots, particularly if few genotypes are planted.

Yield losses as high as 40% have occurred in the most severely diseased fields in Idaho, although losses are generally 10% or less (Forster 1982; Forster *et al.* 1986). In severe cases, 5-10% of the wheat spikes may be sterile due to infection (Forster and Schaad 1988), and the disease may attack a complete nursery so severely that nothing can be harvested (Burton 1931).

Using a modified single tiller approach over three seasons in the USA, Shane *et al.* (1987) calculated that 50% disease severity on the flag leaf resulted in an 8-13% loss in kernel weight and that 100% disease severity on the flag leaf resulted in a 13-34% loss. In Mexico, yield loss in wheat was evaluated in a high rainfall, temperate environment based on infection and yield in single tillers. Data indicated that, on average, losses below 5% can be expected when the percent infected flag leaf area is under 10%. However, up to 20% yield reduction can be anticipated, on average, if 50% of the flag leaf is diseased (Duveiller and Maraite 1993a).

Yield loss is a linear function of the percent infected flag leaf area, and even a small infected leaf area has an effect on yield (**Figure 2.1**). Although the disease is usually observed late in the growing season, the negative effect of the pathogen on yield can be determined as soon as lesions develop on the flag leaf because even a small percentage of diseased leaf area (DLA) has an immediate effect. Since similar effects on yield can be observed when leaves are detached from plants, the effect of BLS on yield is probably related to a reduction in photosynthesis resulting from the extent of DLA (Duveiller and Maraite 1993a).

Based on experimental observations in Mexico's high rainfall, temperate highlands, a formula has been proposed to calculate the expected yield loss based on disease severity at Zadoks' growth stage 73-83 and field incidence. Considering the different percentages of DLA occurring in a field, the expected

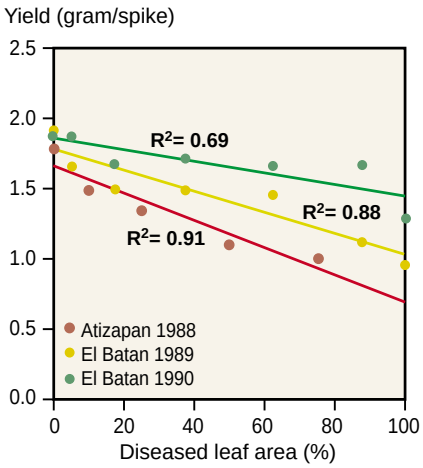


Figure 2.1. Relationship between yield and bacterial leaf streak severity on the flag leaf, at early milk-dough, in genotype Alondra, three years' observation data.

percent yield loss due to *X. t.* pv. *undulosa* in a commercial plot can be calculated as:

$$\text{Yield loss (\%)} = C \frac{\sum_{i}^{n} \text{\% DLA}}{n}$$

where n = number of flag leaves sampled and C = 0.397 on average (Duveiller and Maraite 1993a). A random sample of fertile tillers should be used when applying the formula. For plots of up to 0.004 ha, a sample of 10 primary fertile tillers is suggested. For larger plots and fields, up to 50-80 tillers should be selected at random while walking diagonally across the plot, but sample size is determined depending on disease variability and the desired accuracy (James 1971; Kennedy 1990).

BLS mainly affects grain filling, but grain number was significantly correlated with BLS severity levels two out of three years under Mexican conditions (Duveiller and Maraite 1993a). This confirms a previous report by Waldron (1929) but not results obtained by Shane *et al.* (1987).

In triticale, both yield and test weight may be reduced (Zillinsky and Borlaug 1971). When the average of infected genotypes was compared to the mid-variety in the test, yield losses ranged from 12 to 43% and test weight was reduced from 2 to 13% (Fuentes 1974).

Symptoms

Leaf streak and black chaff

Typical symptoms on the leaf consist of elongated, light brown lesions, several centimeters long, which are initially distinct but later coalesce to cover larger solid areas. Early symptoms are characterized by translucent stripes that are easily seen under incident light. Initially lesions are water-soaked and produce honey-like exudates under humid conditions (Smith 1917; El Banoby and Rudolph 1989; Duveiller and Maraite 1993b). If undisturbed, the exudates harden into yellowish, resinous granules studding the surface of the lesions and are easily detachable (**Figure 2.2 a and b**). Frequently, these droplets coalesce when there is dew, rain or guttation water to form conspicuous milky drops that may later spread over the leaf surface and dry down as thin, grayish, almost transparent flakes (Jones *et al.* 1917) (**Figure 2.3 a and b**). In commercial wheat fields, particularly without sprinkler irrigation, lesions with no exudate can be observed (**Figure 2.4**); this makes it difficult to identify the cause of the symptoms without isolating the pathogen.

Dickson (1956) reported that many leaf lesions start at the apex and extend downward; the

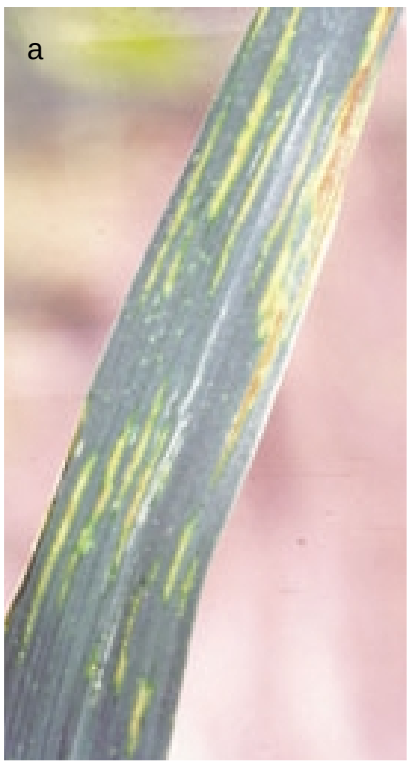


Figure 2.2 a. Bacterial streak of wheat: translucent stripes and exudates. b. Wheat peduncle with resinous granules resulting from severe bacterial leaf streak infection.

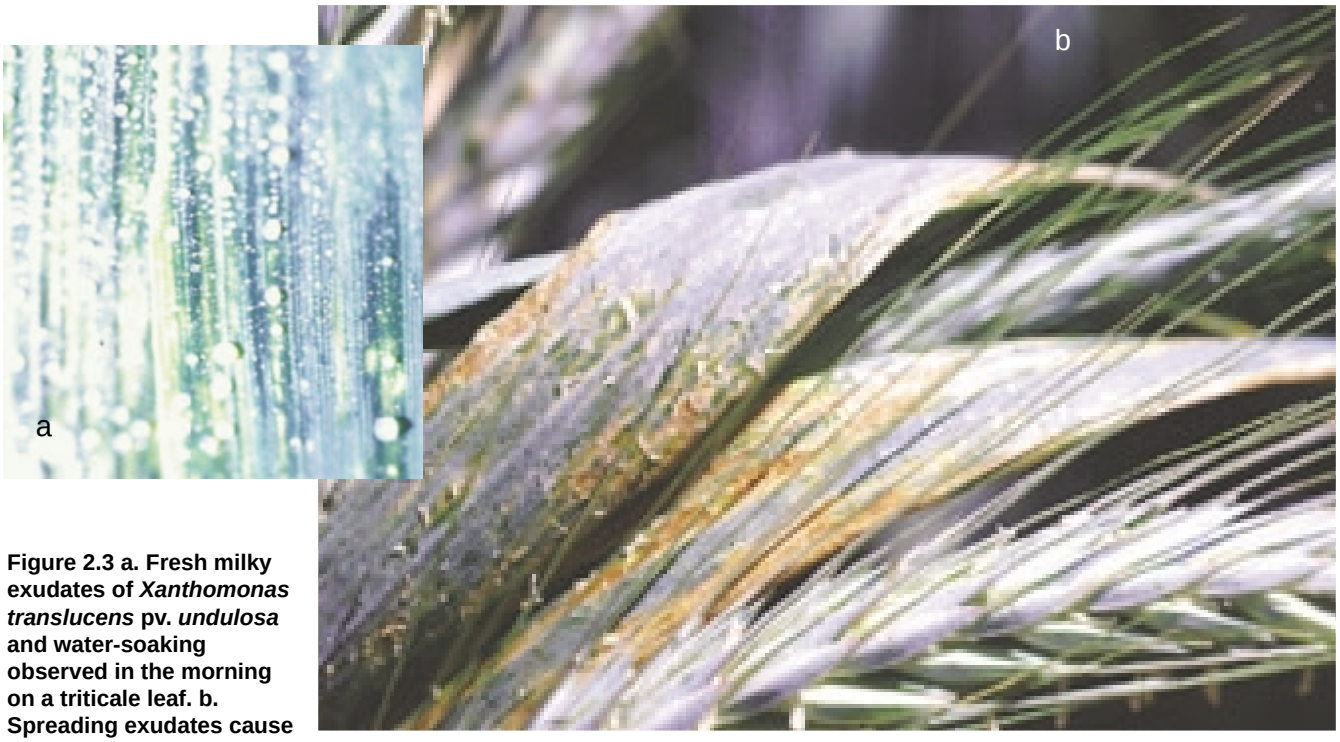


Figure 2.3 a. Fresh milky exudates of *Xanthomonas translucens* pv. *undulosa* and water-soaking observed in the morning on a triticale leaf. b. Spreading exudates cause flakes to form on triticale leaves with bacterial leaf streak lesions.

Figure 2.5. Translucent lesion in the middle of a durum wheat leaf where dew remains longer in the morning.

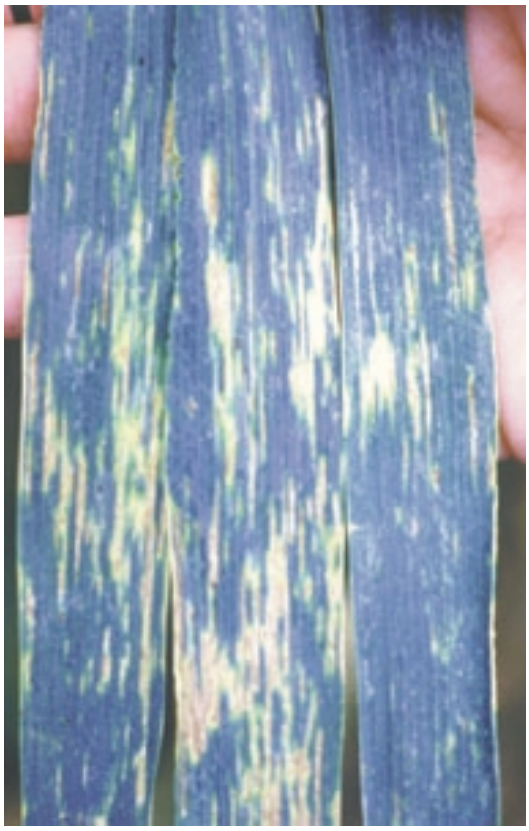


Figure 2.4. Inconspicuous blotches caused by *Xanthomonas translucens* pv. *undulosa* on wheat leaves.

assessment scale proposed by James (1971) suggests similar disease development. This pattern, however, is not typically observed under subtropical field conditions (Duveiller 1994a). Symptoms often develop in the middle of the leaf, where dew remains longer in the morning (**Figure 2.5).** Streaks are more usual on triticale than on wheat.

Culms, leaves, rachis, glumes, and awns may become infected, and symptoms on wheat have been reported to vary with the environment, variety, disease severity, and interaction with fungi (Bamberg 1936; Boosalis 1952). However, extensive experience with different genotypes in Mexico indicates that variations of BLS symptoms on wheat leaves are limited (Duveiller 1994a). Most so-called variations are probably due to melanic reactions on ears and nodes under abiotic stress being mistaken for disease reactions.

Although Forster *et al.* (1986) observed early infection in the field, symptoms often go undetected in seedlings. Bamberg (1936) could not find seedling infection on thousands of plants, and there was no evidence of infection in the field before booting. Similar evidence was obtained in the field and in the greenhouse under Mexican conditions (Duveiller 1994b).

How to distinguish black chaff from brown melanosis

When on the glumes, BLS is characterized by black, longitudinal, more or less parallel stripes that are more numerous and conspicuous on the upper parts (Smith 1917). BLS can be recognized by a greasy appearance

Figure 2.6. Wheat spike showing typical black chaff symptoms: discoloration of the peduncle and alternating bands of healthy and diseased tissue on the awns.

or alternating bands of diseased and healthy areas on the awns (**Figure 2.6**). Purple-black symptoms may extend to the peduncle between the inflorescence and the flag leaf, and may sometimes present a yellow center (Forster *et al.* 1986). On triticale, the bacterium causes moist gray to whitish lesions on the glumes, and discoloration is rarely seen on the peduncle (Duveiller 1989) (**Figure 2.7**).

Several authors have found that susceptibility to melanism on the spike, also referred to as black chaff, is often inherited from stem rust-resistant parents (Goulden and Neatby 1929; Waldron 1929). Bamberg (1936) emphasized the risk of black chaff in progeny of crosses involving the hard red spring wheats H-44 and Hope. Johnson and Hagborg (1944) showed that high temperature conditions, especially when combined with high humidity, favored the development of melanic areas on the glumes, lemmas, peduncles and internodes of rust resistant varieties. As brown

Figure 2.7. Triticale spike infected by *Xanthomonas translucens* pv. *undulosa* showing moist gray lesions on the glumes.

melanosis is known to be associated with the *Sr2* gene for stem rust resistance (McIntosh 1988), it is possible that what early reports of black chaff were really describing was pseudo-black chaff not caused by bacteria.

Sharp discolored interveinal streaks on the glumes suggest the presence of *Xanthomonas*, particularly if also irregularly distributed on the spike, and if there is abundant BLS on the leaves. In contrast, melanosis on the peduncle, which occurs on the same side of most culms in a field as a result of exposure to sunshine (UV light), is indicative of brown melanosis.

Besides this physiological disorder, other very similar symptoms on the spike that are not caused by *Xanthomonas* may be induced by *Bipolaris sorokiniana* (Sacc.) Shoem., *Alternaria* spp., *Stagonospora nodorum* (Berk.) Castellani and E.G. Germano (syn. *Septoria nodorum* (Berk.) Berk. in Berk. and Broome), and *Pseudomonas syringae* pv. *atrofaciens* (McCulloch) Young, Dye and Wilkie 1978.

Epidemiology and Biology

Survival

Seed. Seed is the most important source of primary inoculum, and large-scale transmission of BLS is due to its seed-borne nature (Jones *et al.* 1916, 1917; Smith *et al.* 1919; Tsilosani *et al.* 1969). Depending on storage conditions, it is estimated that the bacterium will die in 63-81 months (Forster and Schaad 1987, 1990). However, in two seed lots infested with 1.3×10^7 and 8.7×10^5 bacteria per gram, respectively, Klykov (1945) (as reported in Neergaard 1977) showed that recovery of the bacterium was reduced by 93% and 79% after only six months of storage, and that more than 99.5% of the bacteria was not detectable after three years.

Black chaff of wheat has a very low transmission rate, i.e., low levels of seed contamination will not result in field disease (Schaad 1988b). In Idaho, more than 60% of all spring wheat seed lots were found to be contaminated, and seed lots with less than 1000 colony forming units (cfu) per gram do not cause field epidemics. This suggests that methods for detecting the pathogen on the seed do not have to be very sensitive (Schaad 1987a; Forster and Schaad 1987). However, the situation may vary from one environment to another, and the pathogen's multiplication capacity should not be underestimated. In the wheat genotype Anahuac, the number of bacteria per lesion increases from about 10^4 to 10^8 in less than 48 h (Duveiller 1992). In the field, seedlings may present symptoms very early in the season, and secondary lesions can start when plants are 3-4 cm (Jones *et al.* 1917; Forster *et al.* 1986). Natural injuries may facilitate the induction of primary lesions on seedlings. Hagborg (1936) and Gorlenko (1941) obtained 81% and 42% infected seedlings, respectively, from artificially wounded seeds infested with the bacterium.

Testing procedures may recover mainly external bacteria, which may not be important for disease induction. So far, the precise location of the pathogen in the wheat seed is not known, but it seems to be located mostly (88.9%) in the external seed coats (Duveiller 1992). Wallin (1946b) intended to show the path the bacterium follows when moving from the seed surface to the aerial portion of the plant. The plumule is infected through wounds or through the stomata on the coleoptile. The pathogen invades the coleoptile, reaches the enclosed foliage, and infects it before the first leaf emerges from the coleoptile.

Soil. *Xanthomonas t.* pv. *undulosa* that survives in soil and crop debris seems not to be a major cause of primary inoculum. The bacterium survives poorly in soil but does better when crop debris is present (Boosalis 1952). Free bacteria cannot survive more than 14 days in air-dried soil and no more than 57 days when infected triticale leaves are mixed into moistened soil (Cunfer 1988). Also, plant stubble usually decays very fast in warm, humid climates, and wheat pathogenic bacteria cannot survive in decomposing debris.

Survival on grasses and overwintering. *Xanthomonas t.* pv. *undulosa* can survive on weeds and grasses due to its broad host range; however, this is probably not significant on annual hosts. In Uruguay, clear streak symptoms caused by *X. t.* pv. *undulosa* were found on canarygrass (*Phalaris canariensis* L.) used as border rows in experimental wheat plots (**Figure 2.8**). Epiphytic populations (i.e., microorganisms living on the surface of the plants) of the pathogen have been detected in Idaho near spring wheat fields on *Poa pratensis*, *Festuca arundinacea*, *F. rubra*, *Hordeum leporinum* and *Medicago sativa* (Thompson *et al.* 1989).

Wallin (1946a) gathered evidence that "*X. translucens*" can overwinter on perennial hosts such as smooth brome (*Bromus inermis* Leyss.) and timothy (*Phleum pratense* L.), which gives the pathogen the opportunity to spread to nearby cereals. The bacterium also seems to overwinter on winter wheat and rye (Boosalis 1952).

Conditions conducive to epidemics

Humidity and temperature. Bacterial leaf streak outbreaks are characterized by sporadic epidemics and higher incidence in breeder's plots (Kamel 1981; Schaad 1987a); they are usually observed by farmers relatively late in the growing season (Forster *et al.* 1986). These factors make gaining a better understanding of the epidemiology of this disease more difficult.

BLS is thought to occur during the wet season or in sprinkler irrigated fields where humidity is high (Forster *et al.* 1986; Bamberg 1936). However, the relative importance of dew, rainfall or irrigation—by sprinkler or gravity—versus such environmental conditions as temperature is not well documented.

Moisture facilitates the pathogen's release from the seed and contributes to leaf colonization and invasion of leaf tissue. Free water allows the pathogen to spread in the field and to disperse on the leaf, thus increasing the number of lesions. Bacteria enter through the stomata and multiply in large masses in the parenchyma. This causes elongated streaks limited by the veins, which act as barriers. Later milky or yellow exudates form on the surface of lesions. Rain and wind greatly influence the spread of these exudates and of the disease from leaf to leaf throughout the field (**Figure 2.9**). However, brief but intense rainstorms, frequently observed in more tropical regions, can also wash the inoculum down the plant. Micro-injuries to awns and leaves caused by hail or wind may contribute to bacterial penetration of the blades.

The BLS-inducing pathogen tolerates a wide range of temperatures (15-30°C) (Duveiller *et al.* 1991) and grows best when temperatures are above 26°C (Forster *et al.* 1986). Recent studies (Duveiller and Maraite 1995) have shown that temperature has a major impact on epidemics. Pathogen multiplication in leaf tissue is directly dependent on temperature, and dry air conditions (< 30%) do not limit disease progress. Symptoms only occur when temperature allows the bacterial population to reach an estimated threshold of 10^8 cfu/leaf (**Figure 2.10**). Low temperatures retard the multiplication of the pathogen and disease progress.

It is therefore not surprising that BLS prevails in warmer, non-traditional wheat growing areas where rainfall is limited but humid night-time conditions (dew) are

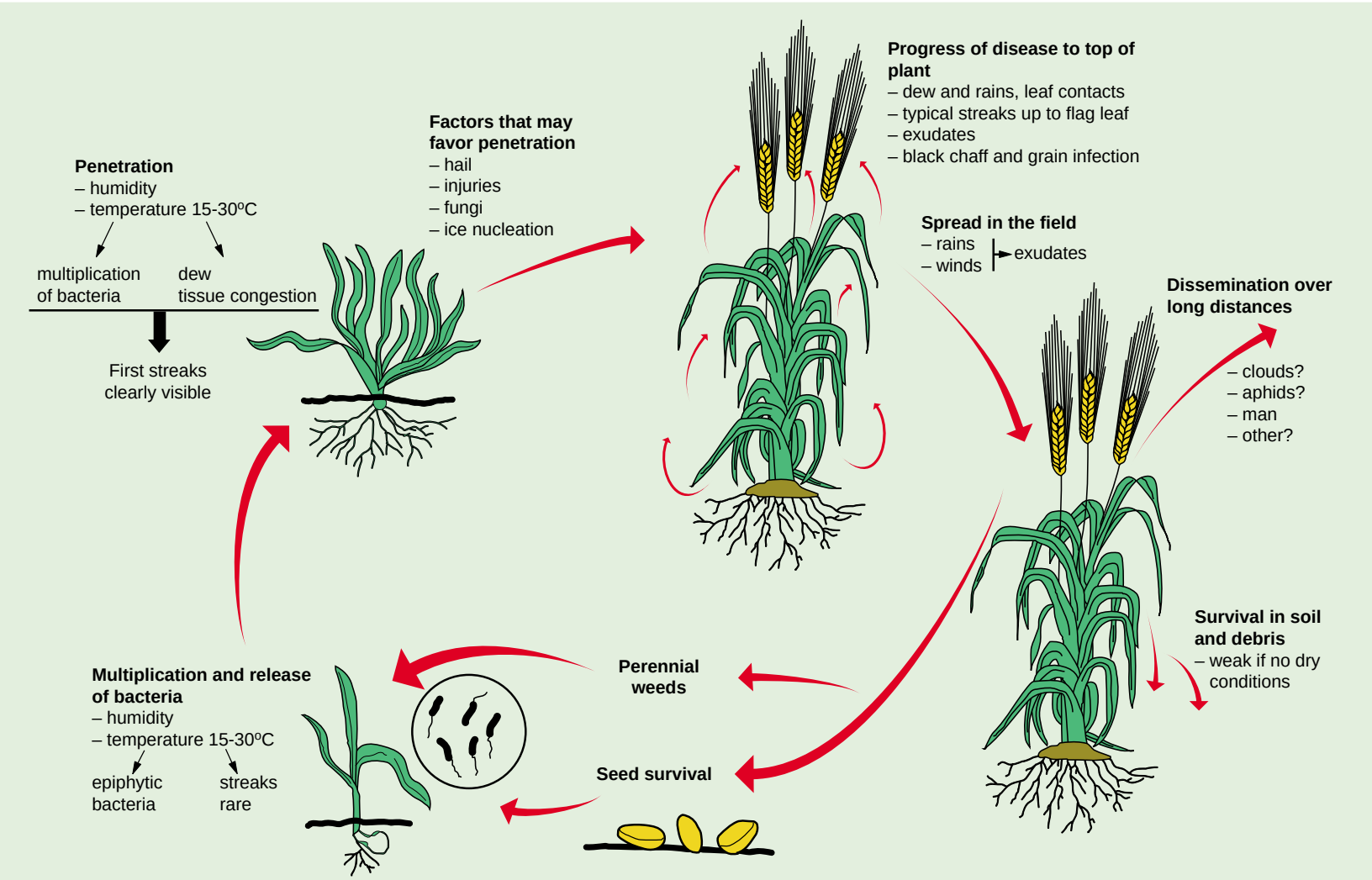
Figure 2.8. Streaks caused by *Xanthomonas translucens* pv. *undulosa* can be found on other Gramineae such as canarygrass.

enough to favor penetration of the parenchyma. Once inside the leaf, the bacterial population can still grow, and leaf moisture does not constrain that growth even under dry air conditions.

Epiphytic populations may be important for understanding the etiology of BLS and discovering why the disease is sporadic. *Xanthomonas t.* pv. *translucens* can multiply and persist on tomato leaves for several weeks, which suggests that survival does not depend on host plant infection and that the bacterium may reside on non-host species (Timmer *et al.* 1987). Forster and Schaad (1988) reported high epiphytic populations on wheat leaves after Zadoks' growth stage DC50, and a detectable level of the pathogen was observed at DC30. In Mexico, it was possible to monitor a *X. t.* pv. *undulosa* population in plots of symptomless genotypes contrasting in their field resistance to the pathogen (Duveiller 1994c). The population of pathogenic bacteria decreased after a heavy rainfall, which suggests that epiphytic *X. t.* pv. *undulosa* are present on wheat leaves before they actually penetrate the parenchyma.

Frost damage and ice nucleation. Bacteria exhibiting the ice-nucleation phenotype have the ability to trigger ice formation at temperatures between 0° and -10°C. Strains of *X. t.* pv. *translucens* express ice nucleation activity at temperatures from -2°C to -8°C (Kim *et al.* 1987; Zhao and Orser 1988). Damage caused to plant tissue by the ice provides conditions suitable for pathogen invasion and multiplication. Frost conditions may thus explain the frequent incidence of BLS in high elevation environments or in regions such as southern Brazil, where wheat is grown during the winter season. Waller (1976) reported that a slight frost precipitated a BLS outbreak in the Toluca Valley of Mexico (2600 meters above sea level) in 1973. However, frost conditions are not common in this area during the summer season when plants present BLS symptoms, and ice nucleation is thus not necessary to induce an epidemic (Duveiller *et al.* 1991a).

Figure 2.9. Disease cycle of *Xanthomonas translucens* pv. *undulosa* and possible ways disease may spread.



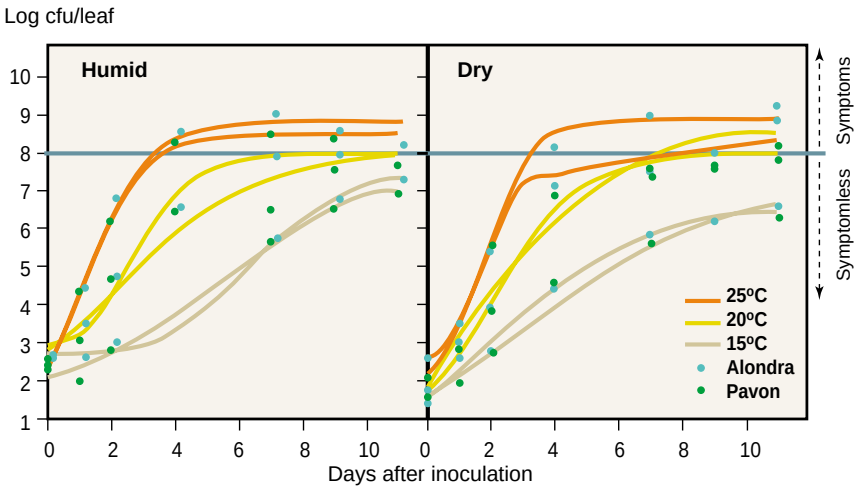


Figure 2.10. Effect of temperature on the multiplication of *Xanthomonas translucens* pv. *undulosa* in wheat genotypes Alondra and Pavon, under humid and dry air conditions; symptoms are visible when the pathogen population reaches 10^8cfu/leaf.

Wheat, barley, maize and bean plants sprayed to runoff with suspensions containing 10^8 cfu/ml of *X. t.* pv. *translucens* suffered greater frost damage than when sprayed with water alone. Bacterial suspensions containing as little as 30 cfu/cm^2 resulted in increased frost injury (Azad and Schaad 1988a).

Interaction with fungal diseases. Wheat with severe root rot infection may be even more infected with "*X. translucens*", which suggests that fungi may have a role in the epidemiology of BLS. Experimental evidence also indicates that root rot and leaf spot fungi, such as *Bipolaris sorokiniana*, predispose wheat to infection by *Xanthomonas* (Boosalis 1952).

Contradictory results have been obtained from studies carried out under greenhouse and field conditions to investigate the interaction between *Septoria nodorum* and *X. t.* pv. *undulosa* (Jones and Roane 1979; Jones *et al.* 1981; Jones and Roane 1981).

It is highly significant that *X. t.* pv. *undulosa* was easily isolated in Mexico from vacuum flasks containing rust uredospores collected in fields where black chaff was present (Duveiller, unpublished). Rust uredospores are used by breeders to infect spreader rows used in selecting for rust resistance. The abundance of sticky exudate and the huge amount of bacteria associated with it facilitate the infestation of rust inoculum. As a result, there is higher incidence of BLS in spreader rows inoculated with rust inoculum if conditions are favorable to the pathogen. This is probably one of the reasons *Xanthomonas* streak problems are observed more frequently in breeding stations.

Spread of the pathogen in the field

Transmission by rain and wind. Pathogen transmission by rain and dew and plant-to-plant contact explains local dissemination (Boosalis 1952). In addition, visitors to demonstration plots, particularly in the morning when dew is at its maximum, increase the spread of bacteria. The disease may spread in the direction of the prevailing wind and driven rain; however, the movement of the disease in space under other conditions proved to be limited. In the USA, in 3-m triticale rows whose borders were inoculated using a grass clipper, diseased plants were found 1 m down the rows but not further down (Cunfer *et al.* 1980). In Brazil, Mehta (1990) indicated that the spread of BLS from one field to another is limited, and disease spread through splashing rain is restricted to distances as short as 4-5 m. This is in agreement with observations made in Mexico (Duveiller 1992).

Role of insects. According to Leach (1940, in Dickson 1956), insects play a role in disease dissemination and infection; however, very little research has been conducted on the topic. Insects may occasionally be trapped in sticky exudates if the latter are abundant (Jones *et al.* 1917). Under favorable conditions such as water-congested tissues, contaminated aphids can transmit *Xanthomonas* to wheat and barley and aid in long distance dissemination (Boosalis 1952). However, the role of aphids in long distance transmission of the disease is probably limited.

The Pathogen

Isolation and identification

Media. *Xanthomonas t.* pv. *undulosa* grows fastest *in vitro* at 28-30ºC. Growth is possible but slower at room temperature (17-27ºC) and will stop at around 36ºC. The bacterium can be cultivated on common media such as nutrient agar, YPGA, GYS, KB and Wilbrink's medium (see Appendix). These culture media are not semi-selective and can be used for a wide range of bacteria. Semi-selective media

include KM-1, XTS and WBC. When no selective medium is available, Wilbrink's medium (Sands *et al.* 1986) is preferred, given the pathogen's typical yellow colony is best distinguished from saprophytes on this non-selective medium (**Figure 2.11**). Wilbrink's medium is particularly useful for massive inoculum production from a pure strain, since it induces an abundant and fast-growing culture.

Pathogenicity tests. Soil inoculation of the pathogen does not induce the disease in cereal seedlings (Hagborg 1936). Seed inoculation using injured, unsprouted grains coated with a bacterial slime or suspension is workable but tedious and unsatisfactory for testing pathogenicity (Hagborg 1936).

Forcing a bacterial suspension into the leaf whorl of young plants (4-5 leaves) or into the boot of older plants with a hypodermic syringe is a very effective inoculation method (Bamberg 1936). This was confirmed at CIMMYT, where plants are usually incubated for five days in a humid chamber after inoculation (Duveiller 1994b). In some cases, water-soaking is observed as early as 3-4 days.

Boosalis (1950) obtained good infections using a partial vacuum. Hagborg (1970) proposed a device for injecting solutions and suspensions into thin leaves of plants. The device consisted of tongue seizing forceps, soft rubber stoppers, and a hypodermic needle and syringe (see **Figure 3.9**). Several injections per minute can be applied with this apparatus, but the size of stomatal openings, the amount of pressure exerted and how long pressure is applied affect the amount of tissue infiltrated. Sun and He (1986) used leaf clippings under conditions optimal for expressing typical symptoms at 22°C. Also, Colin *et al.* (1990) used an inoculation technique where detached leaves are infiltrated to test pathogenicity. However, it is better to use whole plants for inoculation and for pathogenicity tests.

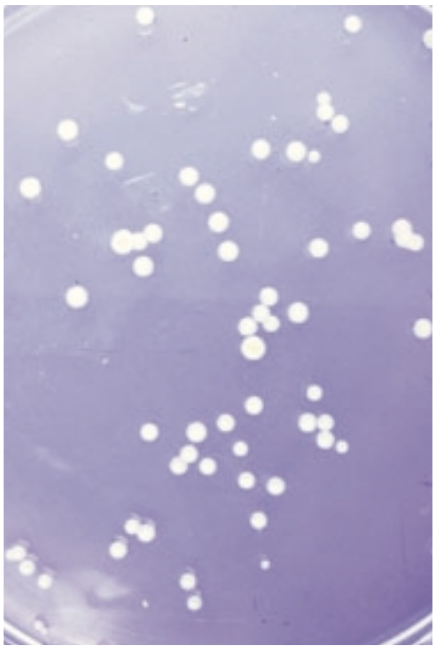


Figure 2.11. Colonies of *Xanthomonas translucens* pv. *undulosa* on Wilbrink's medium present a typical yellow color that easily distinguishes them from saprophytic bacteria isolated from a bacterial leaf streak lesion.

Diversity of *X. translucens* strains that attack wheat and other small grains

Host range and other host/pathogen relationships. Since BLS was first identified on wheat, several pathovar names have been used for *X. translucens* strains isolated from small grains; however, these strains have not always been subjected to differential host range pathogenicity tests. This has caused confusion: in the first place, strains having different names (based on the host plant from which they were isolated) may be similar; second, many authors use the name *X. t.* pv. *translucens* in a general sense for any cereal streak pathogen (Bradbury 1986) although the names of four BLS-inducing pathovars of *X. translucens* are currently included in the most recent ISPP list of plant pathogenic bacteria (Young *et al.* 1996):

Xanthomonas campestris pv. *translucens* (Jones, Johnson and Reddy 1917) Vauterin, Hoste, Kersters and Swings 1995
X. t. pv. *cerealis* (Hagborg 1942) Vauterin, Hoste, Kersters and Swings 1995
X. t. pv. *secalis* (Reddy, Godkin and Johnson 1917) Vauterin, Hoste, Kersters and Swings 1995
X. t. pv. *undulosa* (Smith, Jones and Reddy 1919) Vauterin, Hoste, Kersters and Swings 1995

If the ISPP rules are followed correctly, the pathovar name *X. t.* pv. *translucens* should be reserved for strains pathogenic on barley only (Jones *et al.* 1917) and the name *X. c.* pv. *hordei* should be considered a synonym. This was indicated at the 9th Congress on Plant Pathogenic Bacteria held in Madras, India, in 1996.

Xanthomonas t. pv. *undulosa* designates strains pathogenic on wheat and triticale and can be isolated from several hosts including wheat, barley, triticale and rye. Hence, its host range is not only broader than that of *X. t.* pv. *translucens*, but also covers the host range of *X. t.* pv. *cerealis* as defined by inoculation tests conducted on oat, rye and *Bromus* (Boosalis 1952; Bragard 1996; Bragard *et al.* 1995).

Xanthomonas t. pv. *secalis* has been described as pathogenic on rye (Reddy *et al.* 1924). However, strains with this pathovar name have been reported to infect barley, oat and wheat, although they should not have done so according to the pathovar concept (see Chapter 1) (Bradbury 1986; Cunfer and Scolari 1982; Bragard *et al.* 1995) (**Table 2.1**).

The issue of correctly naming and distinguishing pathovars may appear irrelevant given that different pathovars may induce similar symptoms on the same crop (i.e., wheat). However, studies on pathogenic specialization (Hall and Cooksey 1986; Mellano and Cooksey 1988) are important when searching for disease resistance. Host specific virulence (*hsv*) genes that could expand the host range of *X. t.* pv. *translucens* have been cloned (Waney and Gabriel 1990; Waney *et al.* 1991). Based on RFLP analysis, Bragard *et al.* (1995) showed that strains pathogenic on barley, but not on wheat, clustered in a genetically different group.

Clear differences in aggressiveness were noted among *X. t.* pv. *undulosa* strains from various geographical origins. Whereas typical strains induce extensive stripe symptoms on wheat and barley, strains from some areas induce limited symptoms (Bragard and Maraite 1994). No evidence of strong race specialization has as yet been found on wheat, as indicated by highly non-significant cultivar x strain interaction (Milus and Chalkley 1994).

Biochemical and physiological traits. The BLS pathogen is non-sporing, rod-shaped, Gram negative, and motile by a single polar flagellum. It is further characterized by rods 0.4-0.8 x 1.0-2.5 µm, singly or in pairs, except in peptonized nutrient broth with 2% NaCl, in which long non-motile chains are formed (Jones *et al.* 1917; Dowson 1939).

It is easy to distinguish *X. translucens* from other wheat pathogenic bacteria using several key tests (see Chapter 1). This bacterium is oxidative, i.e., it always produces acid from glucose under aerobic conditions. No nitrate to nitrite reduction is observed (Dye 1962). The reaction for Kovacs' oxidase and arginine dihydrolase is also negative. There is no 2-ketogluconate production, and esculin hydrolysis is positive (Miyajima 1980; Bradbury 1984). Hypersensitivity on tobacco is positive (Mohan and Mehta 1985). Unlike *X. campestris*, *X. translucens* strains do not hydrolyze starch and do not use lactose (Schaad 1987b).

Within the same bacterial species (e.g., *X. translucens*), pathovars group strains that can only be recognized based on host range. Therefore, very few biochemical and physiological tests are useful for differentiating strains at the pathovar level. Hence, metabolic fingerprinting obtained with the BIOLOG MicroPlates™ system and based on the use of

Table 2.1. Comparison of the host range of *Xanthomonas translucens* strains from cereals and grasses after inoculation by water injection plus pricking of wheat (cv. Alondra), barley (cv. Corona) and oat (cv. Alfred) plants at the four-leaf stage.

				Host range		
Strain[a]	Name	Origin	Species where first isolated	Wheat[b]	Barley	Oat
NCPPB2821	*X.t.* pv. *undulosa*	Canada	*Triticum turgidum* var. *durum* L.	+	+	C
UPB480	*X.t.* pv. *undulosa*	Pakistan	*Triticum turgidum* var. *durum* L.	+	+	C
UPB513	*X.t.* pv. *undulosa*	Mexico	X *Triticosecale* Wittmack	+	+	C
UPB605	*X.t.* pv. *undulosa*	Brazil	*Triticum aestivum* L.	+	+	C
UPB645	*X.t.* pv. *undulosa*	Syria	*Triticum turgidum* var. *durum* L.	+	+	C
NCPPB973	*X.t.* pv. *translucens*	USA	*Hordeum vulgare* L.	(+)	+	–
UPB684	*X.t.* pv. *translucens*	Iran	*Hordeum vulgare* L.	–	+	–
UPB780	*X.t.* pv. *translucens*	Spain	*Hordeum vulgare* L.	–	+	T
NCPPB2820	*X.t.* pv. *translucens*	India	*Hordeum vulgare* L.	–	+	C
NCPPB2822	*X.t.* pv. *secalis*	Canada	*Secale cereale* L.	+	+	C
UPB676[c]	*X.t.* pv. *secalis*	South Africa	*Secale cereale* L.	–	+	T
NCPPB1944	*X.t.* pv. *cerealis*	USA	*Bromus inermis* L.	+	+	C

[a] NCPPB = National Collection of Plant Pathogenic Bacteria, Harpenden, England; UPB = Unité de Phytopathologie Bacterial collection, Louvain-la-Neuve, Belgium.
[b] + = positive reaction, compatibility; (+) = weak positive reaction; - = negative reaction; C = chlorosis; T = translucens spot.
[c] Received as pathovar *translucens* from J. Smith, Small Grains Centre, Bethlehem, South Africa.

carbohydrates and amino acids by the pathogen should not be expected to aid in identification at the pathovar level within the same species. Moreover, all biochemical and physiological tests are not of equal taxonomical value; many of them may give variable results for a population of strains of the same pathogen.

Other differential traits. Based on host range and electrophoretic patterns of cell proteins, it was confirmed that *X. translucens* strains collected from wheat and barley in Iran were different (Alizadeh and Rahimian 1989; Alizadeh *et al.* 1996). However, Kersters *et al.* (1989) could not distinguish *X. t.* pv. *undulosa* from *X. t.* pv. *translucens*. Fatty acid profiles do not clearly differentiate between *Xanthomonas translucens* pvs. *translucens*, *cerealis*, *secalis* and *undulosa* (Stead 1989).

Bacteriophages specific to *X. t.* pv. *undulosa* and *X. t.* pv. *secalis*, as well as phages polyvirulent for several "*X. translucens*" strains, were isolated by Katznelson and Sutton (1954). However, their early attempts to use pathovar-specific phages to identify cultures of *X. t.* pv. *undulosa* or *X. t.* pv. *translucens* were unsuccessful because available phages were strain-specific. More recently, a bacteriophage specific to *X. t.* pv. *undulosa* from wheat and triticale, which, in association with other determination tests, may prove to be suitable for quick identification of the bacterium, was obtained from severely infected leaves of the susceptible genotype Alondra (Mohan and Mehta 1985). Similarly, phages that are highly specific to pathogenic strains of *X. translucens* have been isolated from wheat by Forster and Strausbaugh (1994).

Immunological methods do not help to differentiate among the various pathovars of *X. translucens* from cereals. The high degree of serological homogeneity within this group was confirmed in several studies based on polyclonal antibodies (Hagborg 1946; Elrod and Braun 1947a, 1947b; Azad and Schaad 1988b; Samson *et al.* 1989), although Fang *et al.* (1950) grouped five forms of "*X. translucens*" into four serotypes and observed that the immunological closeness between *X. t.* pv. *undulosa* and *X. t.* pv. *secalis* coincided with their pathogenicity. Research on monoclonal antibodies specific to "*X. translucens*" corroborated that pathovars within this group could not be differentiated, which confirms their close serological similarity. However, using these monoclonal antibodies, all strains virulent on wheat, barley, rye and triticale could be differentiated from less aggressive, deviating *X. campestris* strains isolated from wheat (Bragard and Verhoyen 1993).

Control Strategies

Rotations

There is little information on the role rotations play in reducing black chaff epidemics. Since the major source of inoculum is infected seed, rotations may not play a key role in controlling the disease. Straw can harbor viable inoculum from season to season and cause initial infection in the field, but the number of viable bacteria in infested, overwintered straw is reduced when the straw is incorporated into the soil (Boosalis 1952).

At CIMMYT, Mexico, wheat is grown in the highlands during the wet season, and two growing cycles are separated by a six- to seven-month interval during which vetch is grown as a winter crop. *Xanthomonas t.* pv. *undulosa* is able to survive for that length of time on naturally infected straw kept under dry conditions in the laboratory. In the field, wheat straw from the previous season is sometimes found at planting. However, survival of the pathogen seems improbable due to rotation with a non-host crop and its extreme susceptibility to antagonistic bacteria (Schaad and Forster 1985), especially saprophytic fluorescent *Pseudomonas* (Duveiller, unpublished).

Seed health

Detecting the pathogen through seed washing. The best way to limit BLS is to avoid sowing infected seed. A seed wash test on a semi-selective medium after dilution (tenfold) plating (**Figure 2.12**) is the normal non-destructive procedure used in pathogen-free seed certification. The method has the advantage of detecting living pathogens. The number of colony forming units per gram of seed gives an estimate of the number of bacterial cells present in the sample. The number of single colonies growing on the agar medium has to be counted, and representative colonies have to be cloned and proven pathogenic on wheat (**Figure 2.13**). The best estimate is obtained by counting colonies on plates where the number of colonies ranges between 50 and 300. Hence, when *x* grams of seed are washed in *x* ml of saline solution

(w:v = 1:1) and 0.1 ml is plated onto agar medium,

$$\text{cfu/ gram} = n_d \times 10^{(d+1)},$$

where n represents the number of colonies counted on the medium at a dilution fold *d*.

Several semi-selective media have been developed:

- Kim *et al.* (1982) developed the KM-1 medium, which exhibited high selectivity on soil samples and barley leaf debris (see Appendix). Compared to Wilbrink's medium, the plating efficiency (i.e., the number of colonies on semi-selective medium/number of colonies on general medium) ranged from

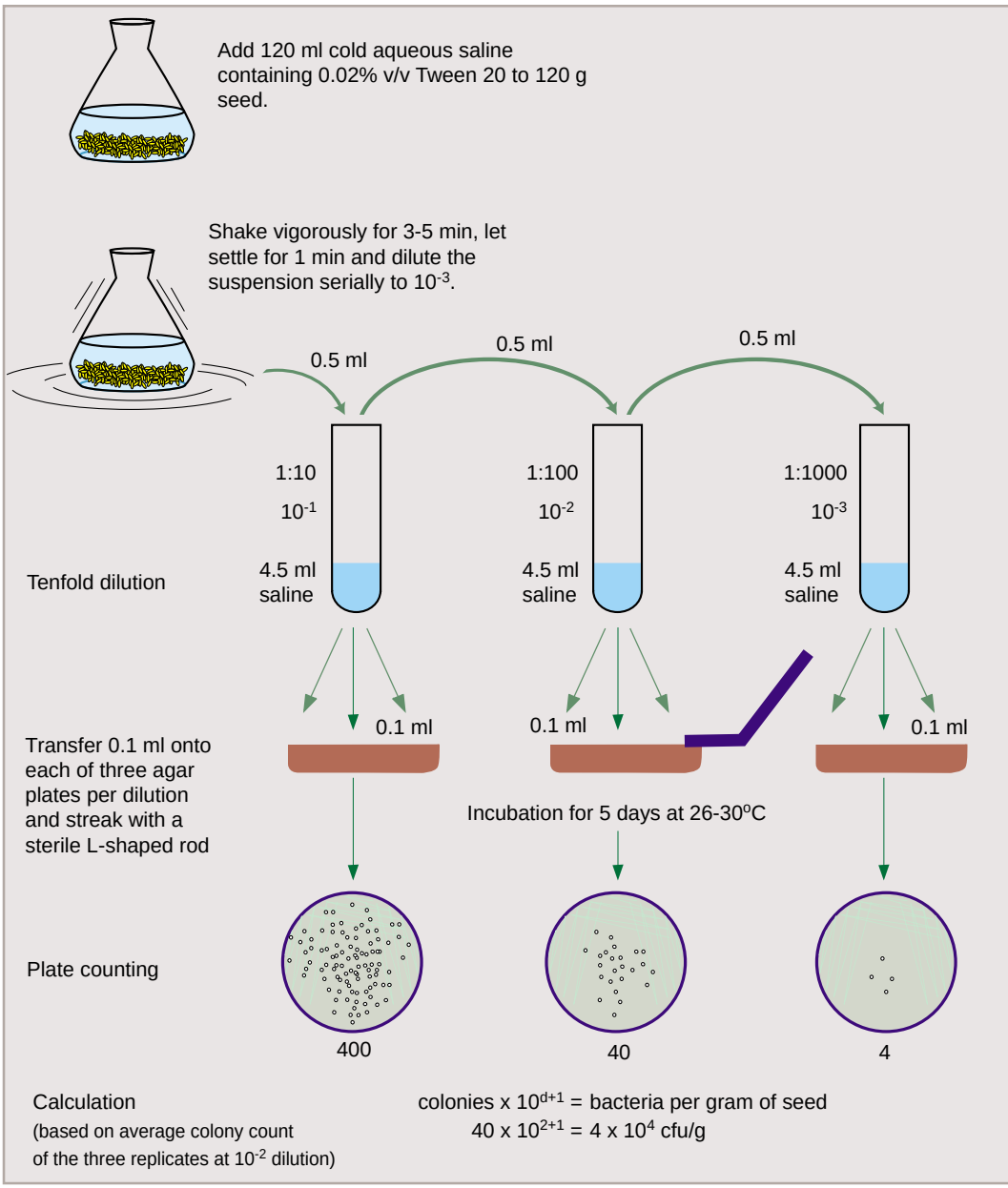


Figure 2.12. Pathogen detection using seed washing and the dilution plating technique.

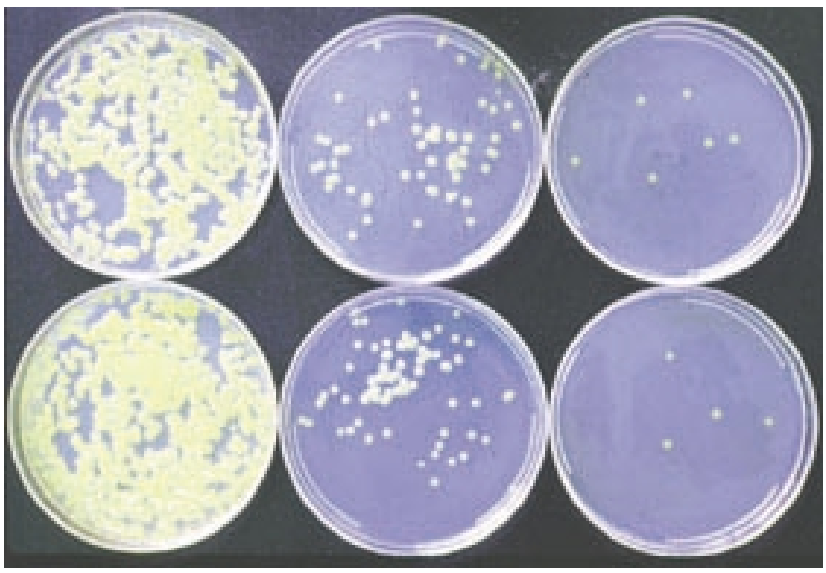


Figure 2.13. Growth of *Xanthomonas translucens* pv. *undulosa* in two replicates of WBC agar Petri dishes after wash water of infected seed was plated and diluted tenfold.

0.91 to 2.13 for strains of *X. t.* pv. *translucens*. However, many "*X. translucens*" strains from Idaho were found to grow poorly on this medium (Schaad and Forster 1985).

- Schaad and Forster (1985) developed the XTS medium (see Appendix) and tested it for isolating the pathogen from wheat seed. To perform the test, take 120 ml of sterile, cold 0.85% NaCl (saline) containing 0.02% v/v Tween 20 and add to 120 g seed (about 3,000 seeds). After shaking vigorously for 3-5 min, let settle for 1 min and prepare tenfold dilutions to 10^{-3} using cold sterile saline. Transfer 0.1 ml of each dilution onto each of three plates of XTS agar and spread with an L-shaped rod. Examine plates after five days' incubation at 30°C. Colonies of "*X. translucens*" are 1-2 mm in diameter, yellow, clear, round, convex and smooth (Schaad and Forster 1993). Streak a known culture onto XTS for comparison. Positive colonies are tested for pathogenicity by injecting a bacterial suspension (approximately 10^5 cfu/ml) into leaf whorls of susceptible wheat seedlings. Disease symptoms appear after incubating for 5-7 days in a dew chamber at 26°C.
- Claflin and Ramundo (1987) used XTS medium with 2 mg/L instead of 8 mg/L gentamycin to increase pathogen recovery. According to Schaad and Forster (1989), antagonistic bacteria may occasionally act synergistically with gentamycin to inhibit the growth of *X. t.* pv. *translucens*. The use of XTS without gentamycin is recommended in such cases. Under Idaho conditions, results of laboratory seed tests using XTS were in agreement with the development of black chaff in the field; levels of 1000 cfu/g or less in seed washes are likely to result in little or no disease (Schaad and Forster 1985). Zero tolerance is not necessary where BLS is endemic (Schaad 1988b), but infested seed should not be used for germplasm exchange.
- Another seed test which proved to be effective under Mexican and other conditions is WBC medium, a modification of Wilbrink's medium (Duveiller 1990b; Duveiller and Bragard 1992; Bragard *et al.* 1993). WBC medium is Wilbrink's medium amended with boric acid (0.75 g/L) and cephalexin (10 mg/L). It does not contain gentamycin but includes cycloheximide to reduce fungal growth (see Appendix). The protocol used in seed washing and dilution plating is similar to the method used with the XTS medium (see above). Duveiller (1990b) pointed out that bacteria recovery may vary among samples from the same seed lot; this sometimes leads to poor correlation between laboratory test results and field detection. The variation could be due either to saprophytic microorganisms that show activity antagonistic to *X. t.* pv. *undulosa* or to uneven distribution of the pathogen among subsamples. As a rule, when using antibiotics, each new flask should be tested prior to utilization, given that antibiotic activity may vary from one lot to another or may be reduced after storage.

Recently Maes *et al.* (1996) developed a method for recognizing BLS-causing *Xanthomonas* pathogens using rDNA spacer sequences and PCR (Maes and Garbeva 1994). The tests proved to be quick (results can be obtained in 5 h, compared to several days using the dilution and plating method) and relatively sensitive (2×10^3 cfu/g of seed), indicating the technique might be useful for detecting those pathogens in seed without isolation. However, this method also detects five other *X. campestris* pathovars with a host range restricted to forage and some ornamental grasses. No data are available on the survival of these grass pathogens on non-host plants, especially in seeds of cereals such as wheat. In addition, there is a risk of false positive PCR detection of dead BLS bacteria.

Serodiagnostic assays. As early as 1939, Gorlenko *et al.* suggested that serological methods could be adapted for detecting black chaff in seed.

Using rabbit polyclonal antibodies for detecting *X. t.* pv. *translucens* in wheat seed, Claflin and Ramundo (1987) were only able to obtain positive readings with a dot-immunobinding assay (DIA) (Lazarovits 1990) when cell concentration was 10^5 cfu/ml or higher. According to these authors, the DIA would be most valuable for identifying *X. t.* pv. *translucens* when used in conjunction with plating on a semi-selective medium.

Frommel and Pazos (1994) used polyclonal antibodies for detecting *X. t.* pv. *undulosa* in naturally infected wheat seed. Using ELISA after enrichment in a semi-selective liquid medium (nutrient broth 8 g/L, glucose 5 g /L, pH 7, gentamycin 5 µg/ml, cephalexin 6 µg/ml, tyrothricin 150 µg/ml, ampicillin 5 µg/ml, cycloheximide 200 µg/ml,

benomyl 80 µg/ml, and Tween 20 at 0.02%), these authors were able to detect the pathogen in samples that originally had less than 5 x 10^2 cfu/ml. However, detection using ELISA without enrichment did not significantly correlate with the potential seedling infection rate determined by growing naturally infested seedlings in the greenhouse.

Bragard and Verhoyen (1993) developed monoclonal antibodies from hybridoma cell lines produced by fusing splenocytes from *X. t.* pv. *undulosa*-immunized Lou rats with IR983F myeloma cells. The monoclonal antibodies reacted positively with *X. t.* pv. *undulosa*, *X. t.* pv. *cerealis*, *X. t.* pv. *translucens*, and

Protocols:

Wash 10 g seed by shaking (200 rpm) in 100 ml sterile distilled water for 30 min at room temperature. Centrifuge to decant debris. Then use either of the following techniques.

Dot-immunobinding assay (DIA)

- Secure a pure nitrocellulose membrane (pore size 0.45 µm) Bio-Rad Trans blot transfer medium that has been previously immersed in TRIS-buffered saline (TBS; 20 mM Sigma 7-9, 500 mM of NaCl, pH 7.5) in a Bio-Dot® microfiltration apparatus (Bio-Rad, Richmond, CA).
- Pipette 200 µl of seed wash water into each well of the apparatus (**Figure 2.14 a**).
- Apply vacuum and wash the membrane with 200 µl of TTBS (TBS 0.05%, Tween 20, pH 7.5) per well.
- Flood the membrane (200 µl per well) for 30 min with a blocking solution (TBS containing 1% bovine serum albumin).
- Add monoclonal antibody AB3-B6 diluted 500 times in the blocking solution (200 µl per well) and let stand for 60 min.
- Wash the membrane twice with TTBS (200 µl per well, eliminated by vacuum).
- Incubate the membrane for 60 min in peroxidase-labeled Mab MARK-PO (IMEX, UCL, Brussels) diluted 500 times in the blocking solution.
- Remove the membrane coated with *X. t.* pv. *undulosa*-(AB3-B6)-MARK-PO, wash with TBS, and expose to solution containing 50 ml TTBS and 30 mg 4-chloro-1-naphtol dissolved in 10 ml methanol.
- The test is positive if a gray precipitate is observed several minutes after adding 50 µl of perhydrol 30% H_2O_2 (**Figure 2.14 b and c**).

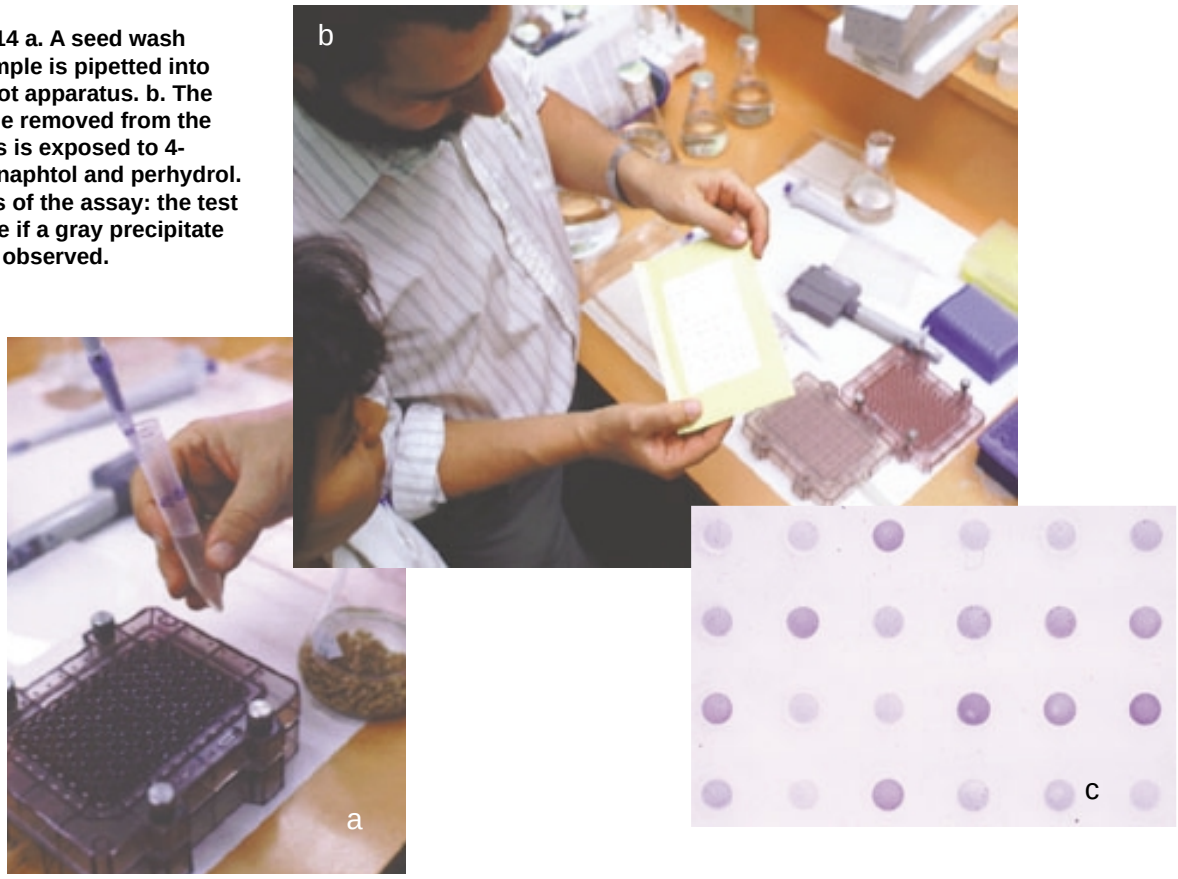
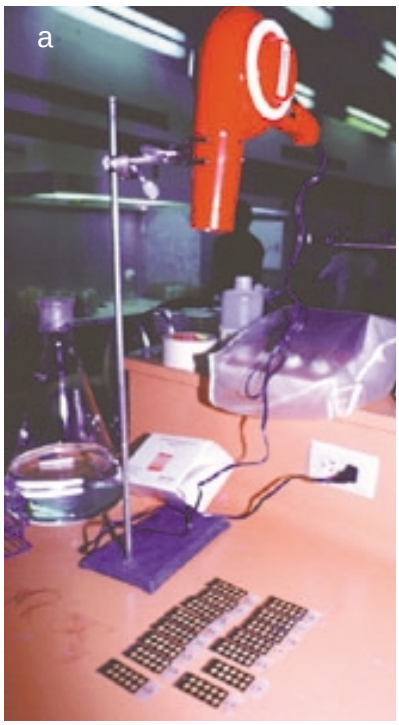


Figure 2.14 a. A seed wash water sample is pipetted into the Bio-dot apparatus. b. The membrane removed from the apparatus is exposed to 4-chloro-1-naphtol and perhydrol. c. Results of the assay: the test is positive if a gray precipitate is clearly observed.

Immunofluorescence (IF)

- Pipette 40 µl of seed wash water directly from the flask into a 6-mm well on a multiwindow slide and then fix with hot air from a hair dryer (**Figure 2.15 a**).
- Expose wells for 60 min to Mab AB3-B6 diluted 100 times in phosphate-buffered saline (PBS; 8 g of NaCl, 2.7 g of $Na_2HPO_4 \cdot 2\ H_2O$ and 1 L of distilled water, pH 7.2); use 20 µl per well.
- Rinse wells with PBS and expose them for 30-60 min in the dark to a mouse anti-rat Mab conjugated with fluorescent isothiocyanate MARM4-FITC (IMEX, UCL, Brussels) diluted 1:100 in PBS. The optimum dilution will vary with each batch of conjugated antibody and therefore must be determined by trying a range of dilutions (usually 1:20 - 1:200 of commercial preparations) on a known positive sample. Use 20 µl per well.
- Rinse with PBS.
- Add three drops of buffered glycerine (100 mg of diphenilamine in 10 ml of PBS, pH 9.6, and 90 ml of glycerol); slip on a coverglass.
- Observe under immersion oil using a microscope equipped with a high-pressure mercury ultraviolet lamp HBO-50 and Carl Zeiss filter combination 10 (x1000) (**Figure 2.15 b and c**). If they cannot be observed on the same day, the multiwindow slides can be stored in the dark for later observation.



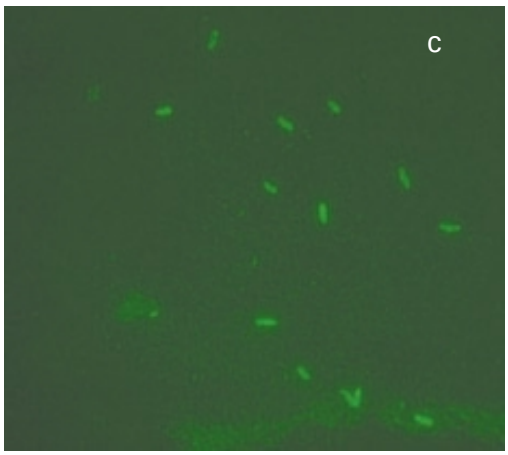


Figure 2.15 a. Seed wash samples are fixed onto multiwindow slides with the help of a hair dryer. b. Epi-UV microscope for the observation of immunofluorescence. c. Fluorescent rods of *Xanthomonas translucens* pv. *undulosa* are observed under immersion oil (x1000).

X. t. pv. *secalis*, and proved more specific than polyclonal rabbit antisera (see Chapter 1).

Serological methods are very useful for identifying strains grown as pure cultures and are also a potential tool for seed indexing procedures. Monoclonal antibody AB3-B6 was used in both immunofluorescence (IF) and DIA to detect pathogens in aqueous seed extracts. These techniques were compared to dilution plating of a seed wash (see p. 37). Seed lots contaminated with a high (>10^4 cfu/g) population of bacteria were consistently identified with all three methods. Immunofluorescence was more sensitive than DIA and gave more reproducible results. The DIA method is simple and requires inexpensive equipment, but the detection threshold is high (10^5 cfu/ml), making it more appropriate for the identification of pure strains. Also, it is not easy to recognize false positive reactions due to dust associated with the seed. On the other hand, although IF requires more expensive equipment, it is relatively quick and sensitive. The detection threshold is 10^3-10^4 cfu/ml. IF positive seed lots should not be used for sowing in areas that favor disease development, since a pathogen concentration of 1 x 10^3 cfu/g of seed is likely to induce an epidemic (Duveiller and Bragard 1992).

Techniques using seedlings. Mehta (1986b) obtained infected seedlings after growing naturally infected seed at 22-25°C, in 20 x 2-cm tubes containing 20 g of sterilized soil and 4 ml of water.

Determining the percent infected seedlings after growing naturally contaminated seed on sterile soil in a moist chamber (100% HR; 22 ± 3°C) did not prove workable under Mexican conditions. No symptoms were found on more than 13,000 seedlings grown from heavily infected seed (>10^6 cfu/g) after four weeks' incubation in trays where each seed was put in a 1-cm deep hole in sterile soil (Duveiller 1994b).

The modified injection technique proposed by Mehta to detect the presence of *X. t.* pv. *undulosa* on wheat, triticale and rye seed and *X. t.* pv. *translucens* on barley seed may be used for quarantine purposes (Mehta 1986a and 1990). Shake seeds (20 g) thoroughly for 90 min in 20 ml of sterile saline; remove seeds and inoculate the suspension into 20-day old seedlings with a hypodermic syringe. *Xanthomonas* streak symptoms are assessed 7-12 days after inoculation. This method is recommended when immuno-fluorescence microscopy is not available (Bragard *et al.* 1993).

X-gal. Sands *et al.* (1984) used X-gal (the substrate 5-bromo-4-chloro-3-indolyl ß-D-gluco-pyranoside) (20 mg/L), from which "*X. translucens*" forms a blue dye, to quickly detect contaminated seed (Sands and Fourest 1989). X-gal solubilized in 1 ml dimethyl formamide can be combined (20 mg/L) with Wilbrink's medium, where lactose replaces sucrose, to show blue colonies of "*X. translucens*" after 4-5 days at 28°C. The major problem with this method is lack of specificity, given that several saprophytic bacteria can induce the blue coloration. Also, the solution used in this technique may be harmful to human health.

Seed treatments

Since no pesticide effectively controls the disease in the field, research on chemical control focuses on seed disinfection. However, the disease cannot be controlled by seed treatments alone, although several studies report partial effectiveness of various compounds (Sands *et al.* 1981).

Braun (1920) indicated that seedling infection was greatly reduced by the use of formalin or copper sulfate. However, results of chemical seed treatments are contradictory. It was believed that the high BLS incidence recorded during the 1980s was due to the ban on mercurial compounds (Forster 1982; Duveiller 1989), but as shown by Forster and Schaad (1988), these products proved to be ineffective for controlling the disease. Cupric hydroxide (Kocide SD), non-volatile mercury (Mist-O-Matic) and volatile mercury (Panogen 15) are also unsatisfactory (Jons *et al.* 1982; Forster 1982).

Mehta (1986c) reduced transmission of *X. t.* pv. *undulosa* by 80% with 300 ml/100 kg seed of Guazatine Plus (syn. Panoctine Plus) in an experiment with naturally infected seed of wheat genotype IAPAR-Caete. When the dosage was increased to 350 ml/100 kg, a 24% reduction in germination was observed (Mehta 1986c). The fungicide substantially reduces disease severity in plots sown with treated seed as compared to plots sown with untreated seed. The treatment is effective if applied at least five months before sowing, but usually ineffective if applied a month before sowing. Also, a few heavily contaminated seeds may escape the product during the procedure and remain contaminated (Mehta and Bassoi 1993).

Seed treatment with acidified cupric acetate (0.5%) at 45°C for 20 min significantly reduced the amount of black chaff in the field (Forster and Schaad 1985 and 1988). Bacteria were isolated from both seed wash water and naturally contaminated seed plated onto XTS after treatment with methoxyethylmercury acetate, ethylmercury-toluene sulphonanilide, phenylmercury acetate, cupric hydroxide, calcium hypochlorite, sodium hypochlorite and calcium propionate but not with hot cupric acetate (Forster and Schaad 1988). Stand count can be reduced significantly compared to other treatments when seed treated with acidified cupric acetate is planted; however, this level of phytotoxicity is considered acceptable for a foundation seed health program (Forster and Schaad 1988).

Recently, an old method based on dry heat seed treatment (Atanasoff and Johnson 1920) was proposed for reducing the amount of bacteria in infected seed. Fourest *et al.* (1990) recommend treating the seed at 72°C for seven days. This method allows treating larger amounts of seed, but experiments conducted at CIMMYT indicate that the method is not completely effective, particularly on seed samples larger than 100 g (Duveiller 1992).

It should be pointed out that the detection technique used in assessing bacterial infestation may make it difficult to evaluate a seed

treatment's effectiveness. When seed is washed and the seed wash is plated on selective agar medium, the bacterium may be rinsed out with the wash water and killed by the bactericide. Also, it is not known exactly where bacteria are located in the seed. Most are located in the external parts of the seed, so they can be reached by a chemical compound. However, bacteria located in the internal layers of the seed coat may be unaffected. When pathogen extraction is non-destructive (e.g., seed washing), bacteria associated with the embryo are sometimes not recovered during testing.

The amount of bacteria in the seed, as well as its heterogeneous distribution, may partly explain contradictory results, particularly if the samples used are too small. If a seed lot contains 10^5 cfu/g, a 99% effective bactericide will still allow 1,000 cfu/g to survive, which is the minimum amount needed to cause an epidemic.

Bactericide seed treatments were evaluated in Mexico using naturally infected wheat genotype Alondra, which is very susceptible to BLS (**Table 2.2**). Laboratory tests were carried out using 10-g samples and kernels individually soaked in 3 ml sterile saline. In the field, the percentage of infected plants in plots (5 x 5 m; 3 replicates) sown with treated seed was determined at Zadoks' growth stage DC45. Results indicated that hot cupric acetate, Panoctine Plus (a.i. guazatine 300 g/L and imazalil 20 g/L), formaline and dry heat consistently reduced the amount of bacteria in the seed as determined using the dilution plate method with WBC agar (Duveiller 1990b). This is in agreement with earlier observations by Mehta (1986d), Forster and Schaad (1988) and Fourest *et al.* (1990). However, although the effects of bactericide treatments were significant (P=0.05), control of BLS in the field was not possible (Duveiller *et al.* 1991). When seed with high levels of *X. t.* pv. *undulosa* is used, bacteria surviving the treatments can multiply to reach the threshold for symptom induction (>10^8 cfu/leaf). The incomplete effect of Panoctine Plus and dry heat was confirmed using heavily contaminated seed (100-g samples), but Panoctine Plus was used only a few days before planting (Duveiller 1992) (**Figure 2.16**). Nevertheless, even if not completely satisfactory, seed treatment with dry heat or a product such as Panoctine Plus is recommended.

Seed multiplication in a disease-free area

Clean seed should be used to produce foundation seed, and multiplication should be conducted in a disease-free area under dry

Table 2.2. Disinfection of heavily contaminated seed of genotype Alondra harvested in Toluca, Mexico in 1988. Infection levels compared by seed washing and dilution plating; average percentage (three 20-m^2 plots) of plants from that seed which showed symptoms in the field after booting the following season (1989) in Toluca and El Batan.

Seed treatment	Log cfu/g[1]	% infected plants Toluca	El Batan
Dry heat 72°C, 7 days	BDT[2]	12.0 ab[3]	1.6 b
Rolitetracycline 1%, soaking 4 h[4]	5.1	0.0 c[5]	4.3 ab
Cupric acetate 0.5%, 40°C, 20 min[4]	5.0	7.3 abc	4.6 ab
Bordeaux mixture (1 lb:1 lb:100 gal), 20 min, 40°C[4]	5.7	3.6 bc	6.7 ab
Formaline 0.8%, 1 h[4]	BDT	9.4 abc	9.8 ab
Panoctine Plus, 400 ml/100 kg seed	5.2	12.5 ab	10.0 a
Cupric acetate 0.5%, 45°C, 20 min[4]	BDT	14.2 ab	12.3 a
Kasugamycin 2%, slurry	6.2	22.1 ab	14.6 a
Copac E, 0.5 ml/50 g seed	6.1	11.7 ab	15.0 a
Control	6.5	17.0 ab	15.5 a

1 cfu = colony forming unit.
2 Below detection threshold (dilution 10^3) on WBC agar.
3 F test (arcsin): Toluca = 2.47*; El Batan = 2.48*; LSD (P=0.05); treatments with the same letters are similar.
4 Seed was rinsed after treatment.
5 Data possibly not reliable due to poor emergence.

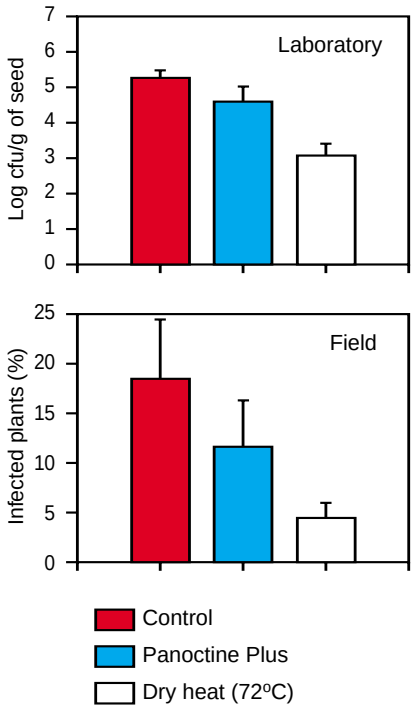


Figure 2.16. Comparison of the bactericidal effects of Panoctine Plus and dry heat (72°C) seed treatments in laboratory and field experiments at El Batan, Mexico, in 1991; the laboratory test was conducted a month before sowing.

conditions without overhead irrigation. Also, it is not a good idea to walk in the fields when leaves are wet. To avoid recontaminating plants produced from clean seed, a distance of 0.4 km is suggested for isolating a seed increase and certification program (Forster *et al.* 1986). Although a seed multiplication field may not show black chaff symptoms, the pathogen may increase on the leaf and head surfaces, resulting in contaminated seed. In contrast, a high percentage of disease in the field does not necessarily result in a higher amount of bacteria in harvested seed lots (Mehta 1990).

Breeding for resistance

Since controlling black chaff through seed treatment is not easy, breeding resistant genotypes appears to be the best way to reduce the risk of yield losses. Screening for resistance is essential for breeding. The material to be screened must be uniformly exposed to the pathogen, and this is only possible through artificial inoculation. Epidemics are sporadic, and natural homogeneous infection in the field is too unreliable to allow adequate evaluation. Lines identified as susceptible under natural conditions may be infected as a result of higher seed infection levels. Also, disease-free genotypes may not really be resistant but may have simply escaped infection.

Greenhouse testing. Greenhouse tests can be conducted on seedlings and young plants by infiltrating low cell concentrations into the leaf. It is important to use low bacterial concentrations to be able to detect measurable differences in resistance. A concentration of 10^4 cfu/ml of a young culture (24 h) on agar medium is usually appropriate. The concentration can be adjusted with the help of a Petroff-Hausser counting chamber (Figure 1.5). A drop of pure bacterial suspension is diluted in water so that a countable number of cells can be observed under the phase contrast microscope (x400). Since living bacteria move, they have to be immobilized to ease the counting. This can be achieved by adding a drop of 4% formaldehyde to the bacterial suspension before observing it in the counting chamber, but the dilution caused by this additional drop must be taken into account.

In another inoculation technique, the seedling pseudostem is filled with sterile water, and then a needle dipped in a young bacterial culture is passed through it. After 5-7 days' incubation in a humid chamber, ideally at 24-26ºC, disease is scored on the emerging leaf.

The major problem with screening at the seedling stage in the greenhouse is the fairly high degree of data variation. To minimize this variation, inoculum concentration, infiltration into confined portions of the leaf blade, and moisture distribution in the dew chamber have to be carefully standardized. Also, the correlation between disease scores on seedling and field data is not always clear, as shown by Duveiller (1992) on a set of 50 genotypes.

Milus and Mirlohi (1994) used a 0-6 scale based on percent water-soaking to evaluate disease reaction on seedlings, whereas Duveiller (1992) measured lesion length and used a 0-4 scale to evaluate the level of exudate production. The correlation between lesion length and degree of exudate production is significant ($P<0.01$) but R^2 is only 0.39. In view of the above, evaluating resistance based on adult plant response in the field is recommended.

Disease evaluation scale (Milus and Mirlohi 1994):

0 = no visible symptoms
1 = chlorosis but no water-soaking
2 = water-soaking less than 10%
3 = water-soaking 10-30%
4 = water-soaking 31-70%
5 = water-soaking 71-100%
6 = water-soaking extended beyond the infiltrated area

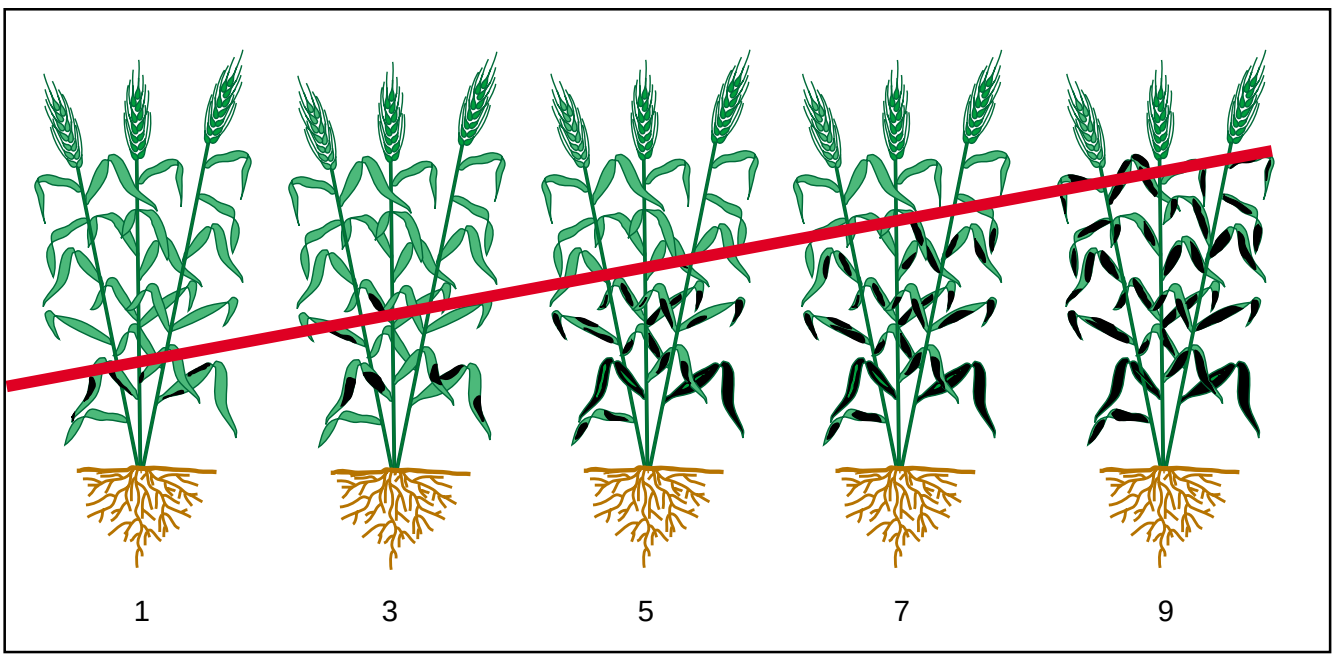
Scale for evaluating exudate production (Duveiller 1992):

0 = no symptoms
1 = water-soaking but no exudate
2 = water-soaking with little exudate
3 = water-soaking with readily detectable exudate
4 = water-soaking with abundant exudate

Field screening. Field inoculation can be done by spraying a concentrated (10^9 cfu/ml) bacterial suspension on plants at the tillering stage. This should be done in the afternoon to take advantage of night-time dew formation, which increases the chances of successful infection through leaf stomata. Approximately 200 Petri dishes containing a pure culture of a single *X. t.* pv. *undulosa* strain cultured on Wilbrink's medium are needed to inoculate half a hectare. After two days' incubation at 30°C, wash the agar and suspend the bacteria in water to produce highly concentrated inoculum (**Figure 2.17 a**). The inoculum can be prepared in the laboratory or in the

Figure 2.17 a. Concentrated inoculum of *Xanthomonas translucens* pv. *undulosa* prepared for dilution and spraying in the field. b. Portable spectrophotometer to adjust inoculum concentration in the field. c. Vial containing bacterial suspension during calibration of concentration.

Figure 2.18. The 0-9 scale proposed by Saari and Prescott (1975) for appraising the intensity of wheat foliar diseases.



field. The concentration can be determined with a portable spectrophotometer at 545 nm (Spectronic Mini 20, Milton Roy Co., Rochester, NY) (**Figure 2.17 b and c**). The aim is to establish the dilution factor necessary to prepare a 10^9 cfu/ml inoculum suspension for spraying. Alternatively, inoculum calibration can be done in the lab by doing a cell count with the help of a Petroff- Hausser counting chamber, or by estimating the number of Petri dishes covered with a 48-h culture that are necessary to prepare the final inoculum. After adjusting the concentration, add Tween 20 (0.02%) to the inoculum to facilitate the spread of the liquid over the leaf. The inoculum suspension (approximately 20 ml/m^2) is applied using a back pack sprayer at 3 lb/cm^2 pressure. Inoculation can be carried out at Zadoks' DC30-35 stage (Zadoks *et al.* 1974; Duveiller 1990a), and may be repeated if necessary.

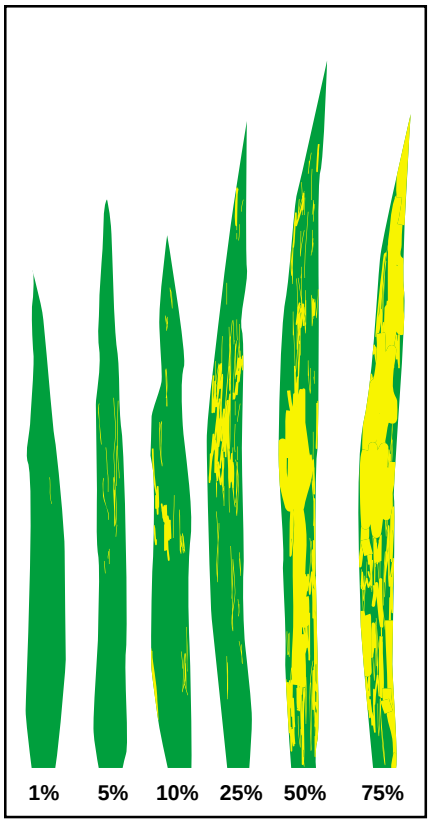


Figure 2.19. Standard disease assessment key showing percentages of leaf surface covered by bacterial leaf streak in bread wheat (Duveiller 1994a).

The disease progresses up a vertical gradient as shown by a smaller damaged leaf area on the flag leaf than on the flag leaf minus one (F-1). The disease progresses upward, and disease severity is assessed at flowering (Zadoks' DC64). The scale proposed by Saari and Prescott (1975) (**Figure 2.18**) for evaluating the intensity of foliar diseases in wheat and barley can be used for screening purposes; it may be modified by adding a second digit for scoring damaged leaf area. This scale is not appropriate for a precise assessment of quantitative resistance or for doing epidemiological studies, for which scales based on actual percent severity are preferred. New scales have been proposed to score severity of leaf damage in wheat and several other small grain cereals such as triticale, barley and rye (Duveiller 1994a) (**Figure 2.19**).

To evaluate disease severity in F_3 populations where a single row represents the offspring of a single F_2 plant, Duveiller *et al.* (1993) considered doing three measurements per row to distinguish segregating lines from non-segregating ones and to identify entries expressing more resistance or more susceptibility than either parent in the cross:

- The lowest and highest severities observed on individual plants within a row were recorded as S_{Min} and S_{Max}, respectively.
- The relative frequency of these two scores was estimated using the following 1-6 scale for assessing distribution of severity within a row:

1 = 1-15% plants with S_{Max} (and 85-99% plants with S_{Min});
2 = 16-35% plants with S_{Max};
3 = 36-65% plants with S_{Max}, indicating more or less equal distribution of S_{Min} and S_{Max};
4 = 66-85% plants with S_{Max};
5 = 86-99% plants with S_{Max};
6 = even severity (no segregation) within the row, $S_{Min} = S_{Max}$.

Disease rating can be done at flowering and again at early dough (Zadoks' DC80). Scores within the disease severity range of either of the respective parents are not considered different from that parent (Figures 2.18 and 2.19).

Immunity does not occur with BLS. Since resistance is incomplete, it is not easily observable under strong disease pressure. Disease may occur even in seemingly resistant parents, provided inoculum pressure is sufficiently strong and the disease has enough time to develop. Although BLS resistance has been identified globally in wheat (Akhtar and Aslam 1985; Bamberg 1936; Boosalis 1952; Thompson and Souza 1989; Duveiller 1990a and 1992; El Attari *et al.* 1996; Hagborg 1974; Milus and Mirlohi 1994; Milus *et al.* 1996), very little information is available on its mode of inheritance (**Table 2.3**).

In the past, resistance to the bacterium was sometimes thought to be controlled by a single genetic factor, *BcBc*, by people who erroneously interpreted and cited

work by Woo and Smith (Nelson 1973). In fact, what Woo and Smith (1962) studied under greenhouse conditions was the inheritance of melanism on the glumes, which they misleadingly referred to as "black chaff." Inoculation with bacteria was not mentioned in the article. Moreover, their study was conducted on a genotype that was highly resistant to stem rust and thus may have carried the *Sr2* gene for stem rust resistance associated with the expression of pseudo-black chaff under stress conditions. Hence, this work was in fact not a study on the inheritance of BLS resistance. Similarly, genetic studies of black chaff resistance by Waldron (1929) and Pan (1940) are misleading because these authors used parents such as Hope and H44 under natural conditions. The observed response was probably caused by pseudo-black chaff and not by the bacterial disease known as black chaff.

Recent research conducted in the field in Mexico involved a full combination of crosses and analysis of data obtained on two dates during an artificially induced epidemic (Duveiller *et al*. 1993). A single strain of *X. t.* pv. *undulosa* was used to reduce pathogen variability and enhance the reliability of this study. Results showed that BLS or black chaff resistance in five wheat lines (Turaco, Alondra, Angostura, Mochis and Pavon) is conditioned by five genes for which the names *Bls1/bls1*, *Bls2/bls2*, *Bls3/bls3*, *Bls4/bls4*, and *Bls5/bls5* have been proposed (**Figure 2.20)**. Genotypes Pavon and Mochis showed the highest level of resistance. None of the five genotypes contained the full set of identified resistance genes, which suggests there are cultivars with more resistance than Pavon and Mochis. It is likely that an accumulation of diverse genes associated with one or more resistance mechanisms will confer increased resistance levels in the field.

A study conducted on triticale indicated the presence of a single dominant gene in each of three BLS resistant lines: Siskiyou, M2A-Beagle and OK 77842. The three genes are either closely linked or the same gene (Johnson *et al*. 1987). The generally higher susceptibility of triticale compared to wheat is not due to the presence of the 6D/6A substitution favored by the empirical selection of complete triticales (Duveiller 1992).

Table 2.3. Wheat genotypes reported to possess resistance to bacterial leaf streak.

Winter wheat	
• Magnum, Bayles, Sawyer Terral 101	Milus *et al*. (1996)
Spring wheat	
• McMurachy Sonora 67/Tezanos Pintos Precoz	Hagborg (1974)
• Sonalika, Blue Silver Jou-Har-79 C-273	Akhtar and Aslam (1986)
• Thornbird	Duveiller (1994)
• Pavon 76, Mochis T88, Nanjing 8331	Duveiller *et al*. (1993)

Figure 2.20. Distribution of postulated genes for resistance to bacterial leaf streak among five bread wheat genotypes (Duveiller *et al*. 1993).

Conclusions

Bacterial leaf streak is a sporadic but widespread disease of wheat that can cause significant losses. The major problem is that the disease is seed-borne. Although zero tolerance of bacteria in the seed is not required due to its low transmission rate, there is a very real possibility that primary inoculum may increase during seed multiplication. The risk of disease is variable in many wheat growing areas of the world, but the possibility of it occurring in areas where it is not usually found should not be overlooked. Fortunately, a specific succession of events is necessary to induce an epidemic. If one of the events required for disease development does not occur, the epidemic may not materialize. Black chaff incidence, severity and distribution may thus vary from year to year, even in disease-prone areas.

Epidemics of bacterial leaf streak may occur in various scenarios. This explains why the disease has a global distribution and is sporadic in areas as different as sprinkler-irrigated wheat fields in the USA, Mexican highlands characterized by marked daytime temperature changes, and the Southern Cone countries of South America, where warm and cloudy days may occur alternately. Because disease occurrence is sporadic, research on epidemiology and resistance is particularly difficult and, consequently, advances in controlling BLS are slow.

Discarding infected seed prior to planting should be the primary control measure, since sowing pathogen-free seed is the first logical step in avoiding an outbreak. Seed indexing procedures are not routinely practiced in many places but should be encouraged. The apparent absence of races and the widespread distribution of the pathogen are not convincing reasons for not implementing seed health procedures to limit the initial inoculum. Foundation seed should be multiplied in disease-free areas where climatic conditions are unfavorable for the development of epidemics. Seed should be disinfected before sowing even if currently available seed treatments are not fully satisfactory. Wheat growers should keep in mind that dry environments do not hamper the multiplication of *X. t.* pv. *undulosa* once it is in the leaf and that temperature has a major effect on pathogen multiplication in leaf tissue.

The most economical and environmentally friendly way of controlling BLS is through genetic resistance, and sources of incomplete genetic resistance have been identified. Differences in the degree of susceptibility are more easily observed in the field in disease-prone areas where artificial epidemics allowing the consistent differentiation between susceptible and resistant genotypes can be induced. Screening for resistance should be encouraged in areas where pathogen populations present the most variation.

Chapter 3 : Wheat Diseases Caused by *Pseudomonas syringae* Pathovars

J. von Kietzell and K. Rudolph

The taxonomy of bacterial pathogens within the *Pseudomonas syringae* group that infects wheat has not been unequivocally defined. Different types of *P. syringae* can induce symptoms on either leaves or spikes of wheat. In this chapter we follow the classification of Bradbury (1986) for identifying them. Pseudomonads that cause the disease known as basal glume rot are designated as *Pseudomonas syringae* pv. *atrofaciens* (McCulloch), Young, Dye and Wilkie 1978, those causing leaf blight are grouped under *Pseudomonas syringae* pv. *syringae* van Hall 1902, and those causing bacterial black node, a disease that produces slightly different symptoms on leaves and spikes, belong to *Pseudomonas syringae* pv. *japonica* (Mukoo) Dye *et al.* 1980.

Distribution

Since the initial description of a wheat disease caused by *P. syringae* in 1920 (McCulloch), wheat pathogens of the *P. syringae* group have been reported from nearly all temperate and subtropical wheat growing regions. The bacteria occur in maritime climates (warm summers, mild winters and constant humidity) and in temperate continental climates (warm, rainy summers and cold winters).

Pseudomonas syringae pv. *atrofaciens* on the spike has been reported in the following states of the USA: New York, Michigan, Kansas, Missouri, Minnesota, North Dakota and South Dakota (McCulloch 1920); as well as in Alberta, Canada (McCulloch 1920); South Africa (Dippenaar 1931; Smith and Hattingh 1991); Mexico (Duveiller 1990c); New Zealand (Wilkie 1973); Australia (Noble 1933); Syria (Maraite, personal communication); Ukraine (Galatchian 1941); Bulgaria (Karov and Vassilev 1981); Germany (Rudolph and Mavridis 1987); Belgium (Maraite, personal communication); and Denmark (Mavridis, personal communication). However, in several cases, symptoms observed on the spike were not specific for basal glume rot. In these cases, widespread epiphytic populations (i.e., bacteria that live on the plant surface but do not induce disease symptoms) of *P. syringae* may have mistakenly been thought to cause spike symptoms.

Disease symptoms caused by *P. syringae* pv. *syringae* on the leaf have been reported in the following states of the USA: Nebraska, North Dakota, South Dakota (Otta 1977), Montana (Scharen *et al.* 1976), and Minnesota (Sellam and Wilcoxson 1976), as well as in the Canadian provinces of Alberta and Saskatchewan (Otta 1974); in Argentina (Teyssandier and Sands 1977); Pakistan (Akhtar *et al.* 1986); South Africa (Smith and Hattingh 1991); and Italy (Varvaro 1983). *Pseudomonas syringae* pv. *japonica* has only been reported in Japan (Mukoo 1955; Oba *et al.* 1990).

Importance

Weather conditions strongly influence disease severity and economic losses. One reason why these diseases have not been investigated thoroughly is that they occur sporadically, and only under extremely humid conditions in spring or summer. This may also explain why studies on the importance of the diseases have often been abandoned after a few years.

In many countries the occurrence of *P. syringae* pvs. *atrofaciens* and *syringae* has only been reported once (Argentina, Australia, New Zealand, Italy and Pakistan) or has not been published at all (Belgium, Ethiopia and Denmark). Consequently, yield losses have never been thoroughly assessed.

These diseases are generally estimated to be of minor importance. Only in a few cases have high losses been recorded, as for example, in South Dakota, where epidemic outbreaks of leaf necrosis were recorded over a period of seven years, during which fields with 75% or more necrotic leaves were common (Otta 1974). In Germany, losses due to

P. syringae pv. *atrofaciens* in an area of marshy soils were estimated to exceed 50% (Mavridis *et al.* 1991). In addition to causing yield losses, *P. syringae* pv. *atrofaciens* infection can severely reduce the grain quality of bread wheat (Vassilev and Karov 1985; Mavridis *et al.* 1991).

Pseudomonas syringae pv. *japonica* is of local importance in Japan only (Oba *et al.* 1990). Although it also infects wheat, the pathogen mainly attacks barley (M. Goto, personal communication 1994). From 1947 to 1955, the disease appeared on a large scale in different regions of Japan, and the damage it caused increased every year during that period (Mukoo 1955).

Symptoms

Spike symptoms

Spike symptoms caused by *P. syringae* pv. *atrofaciens* are generally referred to as basal glume rot (McCulloch 1920). The glumes show a dull, brownish black area at the base (**Figure 3.1 a and b**). Usually only the lower third of the glume, or less, is darkened. Symptoms on the inner surfaces of the glumes are more conspicuous than those on the outside. Sometimes small, water-soaked areas are observed on the margins of the lesions (Toben 1989).

Due to a publication by Wilkie in 1973, atypical symptoms on spikes and stems have often been wrongly attributed to basal glume rot by other authors (Wiese 1987; Smith and Hattingh 1991). In these cases, the entire glume, from the base to the top, shows dark brown or black streaks. Symptoms may develop on a few or all the glumes on a spike, and longitudinal streaks also occur on the upper part of the stem, especially just above the nodes. In some cases, the described symptoms appeared in addition to the typical basal glume rot symptoms (Wilkie 1973). While it is true that *P. syringae* pv. *atrofaciens* has been isolated from plants showing atypical symptoms, it has never been convincingly proved by artificial inoculation that those symptoms were caused by the pathogen. The observed atypical symptoms are also known as brown necrosis (McFadden 1939), melanism or pseudo-black chaff, and can be induced by abiotic stress (Hagborg 1936; Johnson and Hagborg 1944; Sheen *et al.* 1968). Thus it is possible that in the above cases epiphytic pseudomonads may have been isolated by chance and assumed to be the cause of the observed symptoms.

In diseased grain (**Figure 3.2**) symptoms appear at the basal or germ end, varying in color from a scarcely noticeable brown to charcoal black (McCulloch 1920). Although this symptom is typical of *P. syringae* pv. *atrofaciens*, it is not specific and has also been attributed to other pathogens, such as *Bipolaris sorokiniana* and *Alternaria alternata* (Huguelet and Kiesling 1973; Statler *et al.* 1975).

Leaf symptoms

Leaf symptoms incited by *P. syringae* pv. *syringae* are generally referred to as bacterial leaf blight (Sellam and Wilcoxson 1976) or leaf necrosis (Otta 1974). Initial symptoms appear at booting to early heading in the form of numerous, tiny, water-soaked spots on the flag leaf and the first and second leaves below the flag leaf. Within two or three days, these lesions expand and often coalesce into large, grayish-green, desiccated areas (**Figure 3.3 a and b**). These

Figure 3.1 a. Symptoms of basal glume rot caused by *Pseudomonas syringae* pv. *atrofaciens* on wheat spikes; natural infection. b. Symptoms of basal glume rot caused by *P. s.* pv. *atrofaciens* on wheat spikes; artificial inoculation. (Photos: A. Mavridis.)

areas become necrotic and bleach to a light tan or white within a week. Necrosis often progresses until 75-100% of the leaf blade is destroyed (Otta 1974). Ooze has been observed on the lesions in some cases, especially after light dew or rain, on the abaxial side of old water-soaked lesions (Sellam and Wilcoxson 1976, Akhtar *et al.* 1986).

Symptoms caused by *P. syringae* pv. *japonica* (**Figure 3.4**) are known as bacterial black node or bacterial stripe blight. Dark brown lesions appear on nodes and internodes, which later change to a dark, blackish brown color (Mukoo 1955; Fukuda *et al.* 1990).

Epidemiology and Biology

The host range of *P. syringae* pathovars that attack wheat is not precisely known, but appears to be broad. In general, *P. syringae* strains that primarily cause necrosis comprise a large, heterogeneous group possessing a wide host range, in constrast to *P. syringae* pathovars that induce water-soaking as a typical disease symptom (Rudolph 1995). Thus, *P. syringae* pv. *syringae* can infect many different plant species, including lilac, stone fruit trees, bush bean, wheat, sorghum and many weeds (Elliott 1951; Bradbury 1986), and *P. syringae* pv. *syringae* strains from corn, sorghum, foxtail and peach have been found to be pathogenic on wheat seedlings (Otta 1974).

Figure 3.2. Symptoms of basal glume rot caused by *Pseudomonas syringae* pv. *atrofaciens* on wheat grains. (Photo: K. Rudolph.)

Figure 3.3 a. Symptoms of leaf necrosis caused by *Pseudomonas syringae* pv. *syringae* on wheat leaves; natural infection. b. Symptoms of leaf necrosis caused by *P. s.* pv. *syringae* on wheat leaves; natural infection. (Photos: J. Annone.)

Figure 3.4. Symptoms of bacterial black node of wheat caused by *Pseudomonas syringae* pv. *japonica*. (Photos: S. Oba.)

Recently, the basal glume rot symptoms typical of *P. syringae* pv. *atrofaciens* were induced on wheat spikes by *P. syringae* pv. *syringae* strains isolated from lilac, bush bean and *Pennisetum* (see **Table**; von Kietzell 1995). The latter finding supports the assumption that the *atrofaciens* pathovar comprises biovars of pv. *syringae* that have acquired the additional capability of infecting wheat spikes. *Pseudomonas syringae* pv. *japonica* has been reported to be pathogenic on wheat, barley, rye, rice, millets, *Setaria* spp., *Chenopodium* spp. and tomato (Mukoo 1955), but it is not known whether it also causes symptoms similar to those of *P. syringae* pv. *syringae* or pv. *atrofaciens*.

Pseudomonas syringae pvs. *atrofaciens* and *syringae* have important epiphytic phases (English and Davis 1960; Panagopoulos and Crosse 1964; Ercolani *et al.* 1974; Fryda and Otta 1978; Latorre and Jones 1979; Hirano and Upper 1983). For example, in several regions of Germany, *P. syringae* pv. *atrofaciens* was isolated from 10-48% of symptomless wheat leaves and glumes (von Kietzell and Rudolph 1997). Bacterial concentrations reached more than 10^5 cfu / cm^2 in these studies. On barley, the pathogen was isolated from 35-63% of leaf and glume samples without disease symptoms, and bacterial concentrations reached more than 10^7 cfu / cm^2. *Pseudomonas syringae* pv. *syringae* was shown to pass from inoculated wheat seed to the seedling, and to survive as an epiphyte on the first leaves (Fryda and Otta 1978).

In other field experiments (von Kietzell and Rudolph 1997), *P. syringae* pv. *atrofaciens* was found on the first leaves of cereals after seed inoculation. Subsequent leaves were pathogen-free at first, but the bacteria successively invaded the upper leaves and finally reached the flag leaf without causing symptoms of basal glume rot, due to unfavorable weather conditions. Recently, typical *P. syringae* pv. *atrofaciens* strains were isolated from grassy weeds (Fessehaie 1993). The fact that *P. syringae* pv. *atrofaciens* and *P. syringae* pv. *syringae* populations are always present epiphytically on wheat plant surfaces and other hosts indicates that weather conditions are more relevant to disease outbreaks than the presence of inoculum.

Pseudomonas syringae pvs. *syringae* and *atrofaciens* are also commonly found on wheat seeds. For example, in a study conducted by Otta (1977), *P. syringae* pv. *syringae* was found in every one of 21 seed lots representing eight winter wheats from nine locations in the USA and Canada. Similarly, *P. syringae* pv. *atrofaciens* was found on 42 of 54 wheat seed samples and on 35 of 37 barley seed samples that were randomly collected from different cultivars and locations in Germany (Fessehaie 1993; von Kietzell and Rudolph 1996). Bacterial concentrations reached up to 10^5 cfu / 50 g seed in these studies.

Virulence and / or pathogenicity of the bacterium is due to syringomycins and polysaccharides, besides other factors. The syringomycins, cyclic lipodepsinonapeptides (Fukushi *et al.* 1992) produced by all three *P. syringae* pathovars (*P. syringae* pv. *atrofaciens*: Anisimova *et al.* 1994; *P. syringae* pv. *syringae*: Gross and DeVay 1977; Ballio *et al.* 1988; *P. syringae* pv. *japonica*: Young 1992), are phytotoxins with broad antimicrobial activity. Recently, syringomycin E and syringopeptin 25 A were identified in *P. syringae* pv. *atrofaciens* culture fluids (Vassilev *et al.* 1997). Polysaccharides excreted by the bacteria include levan (Sands *et al.* 1988), alginate (Albers 1990; Gross and Rudolph 1987) and lipopolysaccharides (LPS) (Rudolph 1995). The LPS are characterized by an O-specific chain consisting of D-rhamnose tetrasaccharide repeating units with α 1-2 and α 1-3 bonds, and lateral branching substitutes of D-rhamnose, D-fucose or N-acetyl-D-glucosamine (Ovod *et al.* 1995).

Environmental conditions favoring the disease

Since inoculum for basal glume rot and bacterial leaf blight is abundantly present in epiphytic populations, environmental conditions are decisive for disease development. For basal glume rot, periods of extraordinarily humid, cool weather (Toben *et al.* 1991) favored outbreaks of the disease. Also, leaf blight has been reported in years of abundant moisture (Scharen *et al.* 1976) and appeared to depend on the amount of wind-driven rainfall during May and June (Otta 1974). The disease has also been observed in fields with overhead irrigation (Smith and Hattingh 1991).

The Pathogen

Isolation and identification

Epiphytic populations of *P. syringae* pvs. *syringae* and *atrofaciens* are widespread and can be easily isolated from wheat plants. Therefore, the mere isolation of *P. syringae* pathovars does not prove that disease symptoms are caused by *P. syringae* pv. *syringae* or pv. *atrofaciens*. The causal role of the pathogens can be assumed only when high bacterial concentrations occur in the diseased tissue (for example, 10^8 cfu / g fresh weight).

The three wheat-pathogenic *P. syringae* pathovars cannot be differentiated by colony morphology nor by physiological (Otta 1977; von Kietzell *et al.* 1994; Young 1992; Iacobellis *et al.* 1997), serological (Otta 1977; Claflin and Ramundo 1987) and genetic (Iacobellis *et al.* 1997) features. Final characterization is only possible by evaluating symptoms induced on cereals (Toben *et al.* 1989; Maraite and Weyns 1997). Therefore, although the three pathogens can be isolated using similar methods, pathovar identification must include inoculation tests on different hosts.

Media. Many authors have isolated *P. syringae* pvs. *syringae* and *atrofaciens* on King´s medium B (see Appendix). After 24 h, colonies appear that are circular, convex, translucent to opaque and whitish-gray with blue fluorescence that turns green after two days of growth (**Figure 3.5**). No special semi-selective medium has been developed for isolating *P. syringae* pathovars from wheat. However, recent studies have compared many known semi-selective media for isolating *P. syringae* pv. *atrofaciens* (Toben 1989; von Kietzell and Rudolph 1991; Fessehaie 1993). The KBC medium (Mohan and Schaad 1987; see Appendix) shows the highest selectivity for *P. syringae* pv. *atrofaciens* and promotes excellent growth of the pathogen if the concentration of boric acid is reduced from the original 1.5 g / L to 0.5 g / L. Colonies on this medium have the same appearance as on King´s medium B, but growth is slightly slower (**Figure 3.6**).

Isolation from seed. Large numbers of seeds must be analyzed to isolate *P. syringae* pathovars because infestation in seed lots may vary considerably (i.e., 0.2-12.8%, Otta 1977). To isolate *P. syringae* from seed,

Pathogenicity of *Pseudomonas syringae* pvs. *atrofaciens* and *syringae* isolates on wheat spikes.

Strain	Host	Origin	HR-test on tobacco	Symptoms of basal glume rot on wheat spikes
***P. syringae* pv. *atrofaciens* isolates**				
GSPB1440	wheat	New Zealand	+	+
GSPB1723	wheat	Germany	+	+
GSPB1742	barley	Germany	+	+
GSPB1873	wheat	Bulgaria	+	+
GSPB1875	wheat	Bulgaria	+	+
GSPB2073	wheat	South Africa	+	+
GSPB2074	wheat	South Africa	+	+
GSPB2076	wheat	South Africa	+	+
GSPB1576	barley	USA	+	+
GSPB1573	barley	USA	+	+
***P. syringae* pv. *syringae* isolates**				
GSPB1441	wheat	USA	+	-
GSPB1569	barley	USA	+	-
GSPB1570	barley	USA	+	-
GSPB1577	barley	USA	+	-
GSPB1572	barley	USA	+	-
GSPB1574	barley	USA	+	-
GSPB1575	barley	USA	+	-
GSPB2036	barley	USA	+	-
GSPB2067	wheat	South Africa	+	-
GSPB839	sweet cherry	Germany	+	-
GSPB860	sour cherry	USA	+	-
GSPB1004	lilac	Germany	+	+
GSPB1010	lilac	United Kingdom	+	+
GSPB1016	*Pennisetum*	Ethiopia	+	+
GSPB1023	*Prunus*	Yugoslavia	+	-
GSPB1150	*Phaseolus vulgaris*	Germany	+	+

the following procedure is recommended. Soak 50 g of seed overnight in 150 ml sterile saline (0.85% NaCl, 0.01% Tween 20) at 4°C. After dilution series in 0.01 M $MgSO_4$ (2.46 g/L), aliquots (0.1 ml) are plated onto semi-selective KBC medium (von Kietzell and Rudolph 1997; see Appendix). After five days' incubation at 27°C, the number of *P. syringae* colonies per Petri dish is counted. The number of colony-forming units per 50 g of seed is calculated following the formula given for *X. translucens* in Chapter 2 (see p. 36).

Morphology and physiological tests

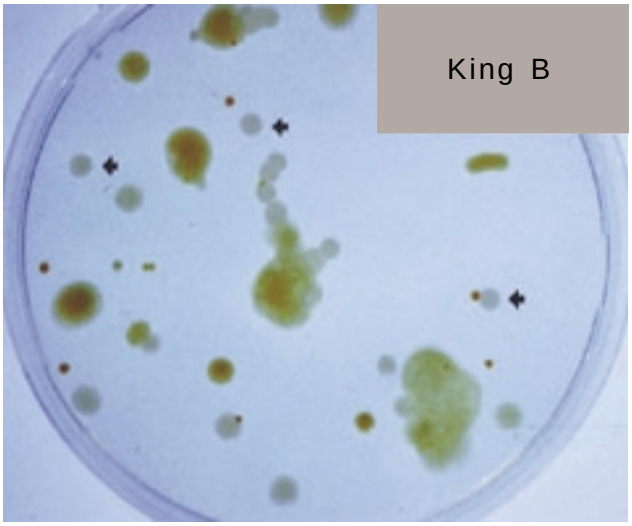
All three pathogens–*P. syringae* pvs. *syringae, atrofaciens* and *japonica*– are straight or slightly curved Gram negative rods occurring singly or in chains of a few cells. These bacteria are motile by a tuft of polar flagella (Bradbury 1986).

The bacteria are strictly aerobic. On King´s medium B they produce a fluorescent blue-green pigment. Tests for arginine dihydrolase and oxidase, and the potato rot test (Sands *et al.* 1988) are negative. Levan is produced on 5% sucrose (Sands *et al.* 1988), and starch is not hydrolyzed (Bradbury 1986). Gelatin, esculin and arbutin are hydrolyzed, and protocatechinate is cleaved at the *ortho* position (Bradbury 1986).

Syringomycin production is determined by a simple biological assay that uses the fungus *Geotrichum candidum* as the indicator organism (Toben *et al.* 1989). Bacterial strains are inoculated as dots onto potato dextrose agar. After five days' growth at 27 °C, the plates are sprayed with dense fungal spore suspensions. As a result of syringomycin production, areas of inhibited fungal growth are recorded after five days.

In BIOLOG-tests (Bochner 1989) (Biolog, Inc.) used to analyze the utilization of 95 carbon sources, positive reactions of *P. syringae* pvs. *syringae* and *atrofaciens* were recorded with the following substances: Tween 40, Tween 80, L-arabinose, D-arabinose, D-fructose, D-galactose, alpha-D-glucose, m-inositol, D-mannitol, D-mannose, D-psicose, D-sorbitol, sucrose, methyl pyruvate, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-gluconic acid, D-glucosaminic acid, DL lactic acid, malonic acid, D-saccharic acid, bromo succinic acid, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-serine, gamma-amino butyric acid, uridine, glycerol, and DL-alpha-glycerol phosphate. The *P. syringae* pathovars *syringae* and *atrofaciens* cannot be distinguished using the BIOLOG (MicroPlates™) system (von Kietzell *et al.* 1994). Young (1992) found that strains of *P. syringae* pv. *japonica* gave reactions identical to those of pv. *syringae* in a large number of determinative

Figure 3.5. Colonies of different bacteria isolated from grain on King's medium B. *Pseudomonas syringae* pv. *atrofaciens* colonies are marked by arrows. (Photo: A. Fessehaie.)



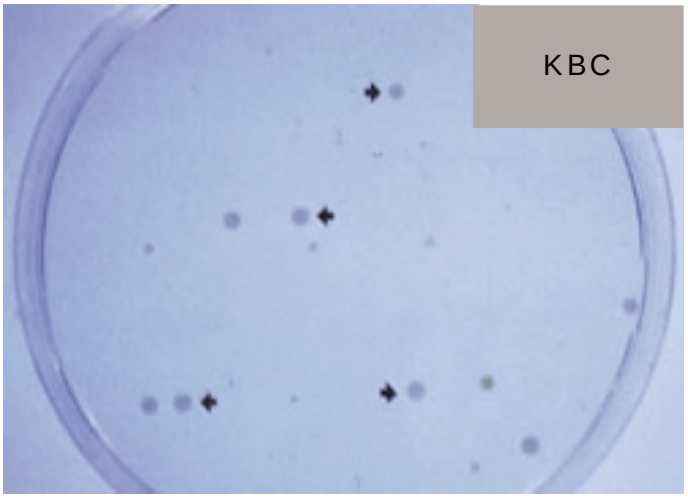


Figure 3.6. Colonies of different bacteria isolated from grain on semi-selective KBC medium; *Pseudomonas syringae* pv. *atrofaciens* colonies are marked by arrows. (Photo: A. Fessehaie.)

nutritional tests and concluded that pv. *japonica* is a synonym of pv. *syringae*.

Pathogenicity tests

The only way to distinguish between these closely related *P. syringae* pathovars is to conduct a pathogenicity test on wheat spikes or a seedling test (see below). Only *P. syringae* pv. *atrofaciens* causes characteristic brownish black margins around inoculation points on barley and wheat seedlings (**Figure 3.7**), regardless of the genotype, and brownish black discoloration at the base of wheat glumes.

Figure 3.7. Seedling test: only *Pseudomonas syringae* pv. *atrofaciens* causes typical dark margins around the inoculation points on barley and wheat seedlings. (Photo: A. Fessehaie.)

A quick pathogenicity test is the tobacco test for hypersensitive reaction (Klement 1963; Klement and Goodman 1967; Klement *et al.* 1990) (see Chapter 1). Slightly cloudy suspensions (more than 10^7 cfu / ml) are injected into intercellular spaces of intact tobacco leaves with a hypodermic needle. (It is easier to inject fully developed leaves than younger ones.) After injection the intercellular spaces become visibly water-soaked for a short time, but the water evaporates and the leaf regains its original appearance within 30 min to 1 h. If the suspension used contains pathogenic bacteria, the injected tissue becomes necrotic within 24 h. Non-pathogenic bacteria cause only a faint chlorosis after a few days. It should be noted that this test does not differentiate *P. syringae* pathovars.

Inoculation procedures for *P. syringae* pv. *atrofaciens*. A quick pathogenicity test on wheat or barley seedlings was proposed by Toben *et al.* (1991). Seedlings are grown on filter paper in sterile Petri dishes. When they are 2-4 cm long, a sterile needle filled with fresh bacterial cells is inserted into the upper part of the leaves. After 2-4 days characteristic brownish black margins will appear around the inoculation point (Figure 3.7). Strains of *P. syringae* that do not infect wheat spikes will not cause symptoms on seedlings.

In testing for pathogenicity on wheat spikes, heads are inoculated at the dough stage (von Kietzell and Rudolph 1991), when spikes are most susceptible to the pathogen. At flowering the spikes are resistant to *P. syringae* pv. *atrofaciens* (Toben 1989), but thereafter susceptibility increases steadily until the dough stage.

To conduct this test, a freshly grown culture is suspended in sterile saline (0.01 M $MgSO_4$), adjusted to an optical density of 0.06 at 660 nm, and diluted to 1:10. This suspension contains about 10^7 cfu / ml and is sprayed with a high pressure sprayer (**Figure 3.8**) or a hand sprayer onto healthy wheat spikes until small droplets cover all surfaces. Inoculated plants are incubated at 90-95% relative humidity and 20-25ºC for 10 days. Since *P. syringae* pv. *atrofaciens* is a weak pathogen, it is very important to maintain these conditions strictly during incubation. After 3-4 days typical brownish black discolorations appear at the base of the glumes. Some wheat cultivars express symptoms of melanism in addition to discoloration at the base of the glumes (Wilkie 1973), but after inoculation with *P. syringae* pv. *atrofaciens,* melanism never occurs without concomitant basal discoloration. Melanism is not a true disease symptom because it can also be induced by abiotic stress (Johnson and Hagborg 1944).

Other authors have described different inoculation procedures. They are less similar to natural infection, but may be preferred if the required wet and cool incubation conditions are not available. Wilkie (1973) placed cells directly from the agar surface onto immature glumes and leaves, and punctured the base of each glume or several parts of the leaf blade with a sterile needle before incubation in a moist chamber. Vassilev and Karov (1986) injected a bacterial suspension (10^7 cfu / ml) into the cavity around the spike that is formed by the flag leaf 7-10 days before heading (Zadoks' DC39-45). Unspecific symptoms developed on the spike, and in some cases the spikes rotted without heading. However, this method has the advantage that controlled chambers are not needed for incubation, since the inoculum is protected against desiccation by the surrounding flag leaf.

Inoculation procedures for *P. syringae* pv. *syringae*. For seed inoculation, wheat seed is placed in a bacterial suspension (10^7 cfu / ml) in a vacuum (produced by a water aspirator) for 5 min and then air dried (Fryda and Otta 1978).

Different methods have been described for leaf inoculation. Bacterial suspensions are sprayed on 20-day-old wheat seedlings and on flag leaves of young adult plants using air brushes (Sellam and Wilcoxson 1976). Inoculated plants are placed at about 20°C in a moist chamber where plant surfaces are kept continuously wet. After three days, small, water-soaked, grayish-green lesions appear on the leaves of seedlings and adult plants. This method best mimics natural infection conditions, but since *P. syringae* pv. *syringae* is a weak pathogen, for the most part only limited and irregular disease symptoms are obtained with spray inoculation (Shane and Baumer 1987). Therefore, a modified Hagborg inoculation technique (Hagborg 1970) has been proposed (Shane and Baumer 1987; Smith and Hattingh 1991). Bacterial suspensions are infiltrated into intercellular spaces of leaves using a syringe with the needle inside a rubber stopper (2.0 x 0.5 cm; **Figure 3.9**). Plants are incubated in growth chambers at 18-20°C with daily misting.

For a quick pathogenicity test, sorghum can be used as an alternative host plant, since it can be inoculated at a younger stage and produces very distinctive symptoms (Otta 1977). Two-week-old plants are vacuum-infiltrated with 10^5 cfu / ml, incubated in a mist chamber for 24 h and then placed on a greenhouse bench at 24-27°C. After one week distinctive spots appear on the leaves.

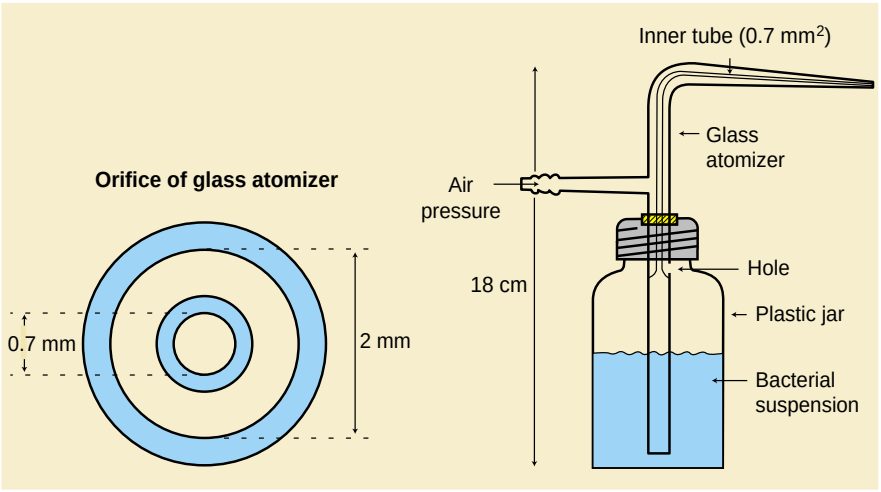


Figure 3.8. Glass atomizer for spray inoculation (from Hokawat and Rudolph 1991).

Seed transmission

Seed infestation can play an important role in disease epidemiology. For example, in field experiments using seed previously inoculated with a spontaneous mutant of *P. syringae* pv. *atrofaciens* that was resistant to rifampicin and streptomycin (von Kietzell 1995), or with a special pv. *syringae* serotype (Fryda and Otta 1978), the inoculated bacteria were found on leaves of the resulting plants. Besides the labelled bacteria, wild-type strains from other sources were identified on symptomless leaves. These results demonstrated that many other sources of inoculum may be present during the growing season.

The systemic invasion of wheat seeds by *P. syringae* pv. *japonica* was shown by histological studies conducted by Fukuda *et al.* (1990). The bacteria first infected the palea and lemma, then passed through the funiculus to invade the caryopsis, and multiplied in its intercellular spaces.

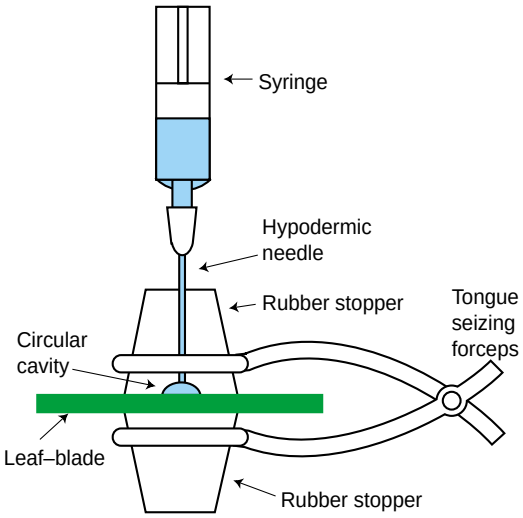


Figure 3.9. Device for injecting bacterial suspensions into thin leaves (from Hagborg 1970 and Klement *et al*. 1990).

Control Strategies

Since all three *P. syringae* pathovars that attack wheat are weak pathogens, control measures have generally not been necessary. In most countries the diseases have been observed only under extremely humid conditions and have not been apparent in subsequent years when weather conditions were not as extreme. As a result, routine control measures have not been established.

Since contaminated seed may play an important role in inoculum transmission, seed lots from heavily infested fields should not be used for sowing. Seed dressings that effectively sanitize seed after bacterial infestation are not available. Management practices may be used as a means of control. For example, after a severe *P. syringae* pv. *syringae* greenhouse infection destroyed all wheat plants (Kimber 1987), repetition was avoided by reducing the peat content and improving drainage of the compost.

Generally, resistant cultivars are an important means of controlling bacterial diseases. For this reason, several investigations were conducted to determine wheat cultivar resistance (Sellam and Wilcoxson 1976; Akhtar *et al.* 1986; Shane and Baumer 1987; Toben *et al.* 1991). Unfortunately, not enough data have accumulated from naturally infected wheat fields to verify these results, which were obtained mostly in growth chambers. On the other hand, field inoculations are difficult to do because the required extreme environmental conditions cannot be mimicked in the field. For example, enclosure of single spikes or whole plants in plastic bags may increase humidity to the optimal level, but may also generate extremely high temperatures. The thermal death point of *P. syringae* pv. *syringae* is 48-51°C (Elliot 1951).

Available data indicate that the degree of susceptibility among wheat cultivars varies considerably. Nevertheless, it is very unlikely that cultivars with complete resistance to several *P. syringae* biotypes will be found, since the pathogen is characterized by an extraordinarily broad host range.

Conclusions

Three pathovars of *Pseudomonas syringae* induce symptoms on either leaves or spikes of wheat: *P. syringae* pv. *atrofaciens* causes basal glume rot on spikes, *P. syringae* pv. *syringae* causes leaf blight, and *P. syringae* pv. *japonica* is the causal agent of bacterial black node on leaves and spikes. These pathogens have been reported from nearly all wheat growing areas in temperate and subtropical zones. Disease outbreaks occur sporadically, usually under extremely humid conditions during spring and summer. Only in a few cases have significant losses been recorded.

Pseudomonas syringae pvs. *syringae* and *atrofaciens* have important epiphytic phases. Populations of both pathovars are always present epiphytically on wheat plant surfaces and on other hosts. Therefore, weather conditions are more relevant for disease outbreaks than the mere presence of the inoculum.

Symptoms on the glumes similar to those of basal glume rot may be caused by other factors such as melanism or pseudo-black chaff due to abiotic stress. In the latter case, epiphytic populations of *P. syringae* pvs. *syringae* and *atrofaciens* may be erroneously identified as causing symptoms on spikes and leaves. However, the mere isolation of the pathogens does not prove that the observed disease symptoms are caused by pathovars *syringae* and *atrofaciens*. These pathogens can be assumed to have a causal role only when high bacterial concentrations occur in the diseased tissue (for example, 10^8 cfu / g fresh weight). No control measures for routine application have as yet been established. However, seed lots from heavily infested fields should not be used for sowing.

Chapter 4 : Other Plant Pathogenic Bacteria Reported on Wheat

L. Fucikovsky and E. Duveiller

This chapter focuses on diseases that are reported on wheat under natural growing conditions, but generally occur in highly specific or rarely observed situations. Some of these diseases are found on widely varying host plants and may be caused by the less specialized bacterial pathogens that constitute the epiphytic flora of plants. Or they may be caused by opportunistic organisms that are favored by unusual conditions but do not normally pose a threat to the wheat crop.

Due to their sporadic occurrence, the bacterial diseases reported in this chapter have not been studied extensively, and little information is available on their control or on the environmental conditions that favor their occurrence. Indeed, some may question the relevance of focusing on diseases that could be considered oddities. Nonetheless, the authors believe they should not be overlooked, since disease problems considered unimportant today may become economically significant in the future as a result of evolving cropping conditions or varietal changes. However, readers should not expect to see the symptoms caused by these pathogens very often.

Bacterial Diseases Caused by Clavibacters

Spike blight caused by *Clavibacter tritici*

Spike blight, also called yellow ear rot or yellow slime rot, was first described in India as tundu on wheat, barley and *Phalaris minor,* with reported infection levels of 60.8, 7.3 and 3.8%, respectively (Paruthi and Gupta 1987). Spike blight is actually a disease complex that includes *Clavibacter tritici* and the nematode *Anguina tritici,* which produces seed galls (ear cockle) in some varieties (Paruthi and Bhatti 1985). It is characterized by a bright yellow gum on the leaf surface of young plants and aborted spikes (**Figure 4.1**). When they emerge, spikes are narrow and short, grains are filled by the bacterial mass and the culm becomes deformed (Swarup *et al*. 1993). The larvae and galls (cockles) of *A. tritici* are vectors of the bacterium (Gupta and Swarup 1972). On GYCA medium (see Appendix), bacterial colonies appear bright yellow, glistening and moist. The color turns orange as colonies age (Swarup *et al*. 1993). Zgurskaya *et al*. (1993) proposed renaming *C. tritici*, *Rathayibacter tritici.*

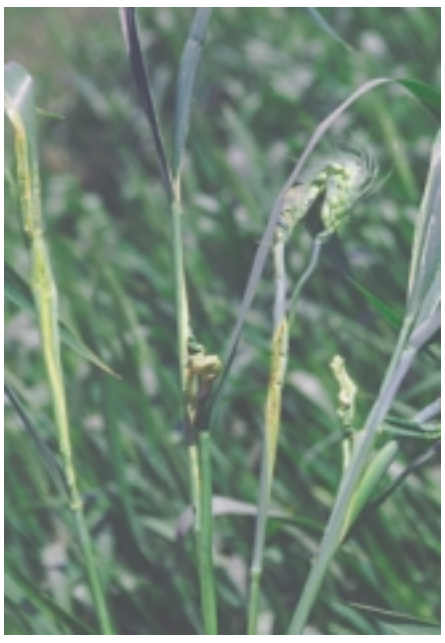


Figure 4.1. A deformed culm and sticky exudate on the spike are symptoms typical of infection by *Clavibacter tritici,* a bacterium associated with bacterial spike blight.

Ear cockle has been reported on five continents. It has become rare in most wheat growing areas but can still be found in Australia (Riley and Reardon 1995), China, Cyprus, Iran (Bradbury 1986), India, Ethiopia, Egypt (Swarup *et al*. 1993) and Pakistan (Akhtar 1987). Bacterial gummosis of wheat was observed in Western Australia (Carne 1926). The bacterium associated with it was not isolated, but only presumed to be *C. tritici*. It may have been *C. toxicus*, a pathogen that produces toxins and affects grazing animals by causing symptoms called flood plain staggers. Associations of *C. toxicus* and *A. tritici* have been produced *in vivo* (Riley 1992), and non-toxigenic *Clavibacter* spp. such as *C. tritici* may be valuable for biological control of *C. toxicus* (Riley 1994). It has been suggested that the disease in India could be controlled by seed and soil treatments using aldicarb sulfone or soil treatment with furadane (Jain and Sehgal 1980).

Removing galls from contaminated seed eliminates the disease. Galls are easily recognized by their dark color. A great number of galls are removed when the chaff is discarded during threshing. Soaking the seed in 20% brine (NaCl or KCl) is very effective because galls float and can be skimmed off, while the seed settles on the bottom. After soaking in brine, wheat seed should be washed 2-3 times to avoid reducing germination. The seed can then be soaked in water for 2 h and heated at 51ºC for 30 min (Limber 1938). The hot water first reactivates and then kills dormant nematode larvae (Swarup *et al*. 1993). Two additional control measures are crop rotation with a non-Gramineae species for two or three years and the use of nematicides.

Bacterial mosaic caused by *Clavibacter michiganensis* subsp. *tessellarius*

Bacterial mosaic was first described in Nebraska by Carlson and Vidaver in 1981 (Carlson and Vidaver 1982a), who found it was widespread but sporadic on numerous wheat cultivars (**Figure 4.2**). Although the causal agent, *C. m.* subsp. *tessellarius*, also attacks triticale (McBeath *et al*. 1981), it is wheat specific but closely related to *C. m.* subsp. *michiganensis* and *C. m.* subsp. *nebraskensis*, which attack tomato and maize, respectively. This specificity is characterized by the population levels of the different bacteria on their respective host.

Bacterial mosaic is characterized by small, yellow lesions with undefined margins more or less uniformly distributed over the whole leaf. Individual lesions may resemble the hypersensitive reaction to rust. Water-soaking and bacterial oozing from lesions are not observed.

Bacterial mosaic caused by *C. m.* subsp. *tessellarius* has been found in Illinois (Chang *et al*. 1990), Alaska, Iowa and Canada. Its economic importance is unknown (Carlson and Vidaver 1982a). However, since it can destroy flag leaves of severely infected plants, the disease may cause significant losses (McBeath 1993).

The bacterium is rod shaped, up to 2 x 0.5 µm, Gram positive and non-motile (McBeath *et al*. 1981). It produces apricot-orange colored colonies on *C. nebraskensis*-specific CNS medium (see Appendix). Based on polyacrylamide gel analysis of cellular proteins, *C. m.* subsp. *tessellarius* was found to be distinct from *C. tritici* and *C. iranicus*, which induce gumming of the inflorescence of wheat (Carlson and Vidaver 1982b).

Clavibacter michiganensis subsp. *tessellarius* is seed-borne (McBeath and Adelman 1986; McBeath *et al*. 1988). Seed infection can be detected by growing longitudinally cut kernels on CNS agar (see Appendix). The kernel halves are put cut-face down on the agar. Production of orange colonies after a 10-day incubation period at 20ºC on CNS suggests the pathogen is present.

Control methods should include discarding contaminated seed and developing more resistant genotypes. There seems to be a wide range of host response to the disease among spring wheat genotypes, which suggests that genetic improvement of this trait is feasible.

Gumming disease caused by *Clavibacter iranicus*

This pathogen has only been reported in Iran (Scharif 1961), where it induces a gumming disease on wheat spikes. The current name of this bacterium is *Clavibacter iranicus* (ex Sharif) Davis, Gillaspie, Vidaver & Harris 1984, brought back into use by Carlson and Vidaver in 1982 (Carlson and Vidaver 1982b). Recently, Zgurskaya *et al.* (1993) suggested renaming this pathogen *Rathayibacter iranicus*. It should be considered as being different from *C. tritici*. The economic importance of this wheat specific disease is not known.

Figure 4.2. Bacterial mosaic caused by *Clavibacter michiganensis* subsp. *tessellarius*. (Courtesy A.K. Vidaver.)

Clavibacter iranicus does not grow on CNS medium (see Appendix) and shows poor growth on nutrient agar, which should be amended with 5% sucrose. Maximum growth temperature is 30°C. This strictly aerobic pathogen produces acid from glucose and trehalose but not from meso-inositol. It tests negative for levan production, nitrate reduction and oxidase, and hydrolyzes esculin. No control measures have been reported.

Bacterial Diseases Caused by Pseudomonads

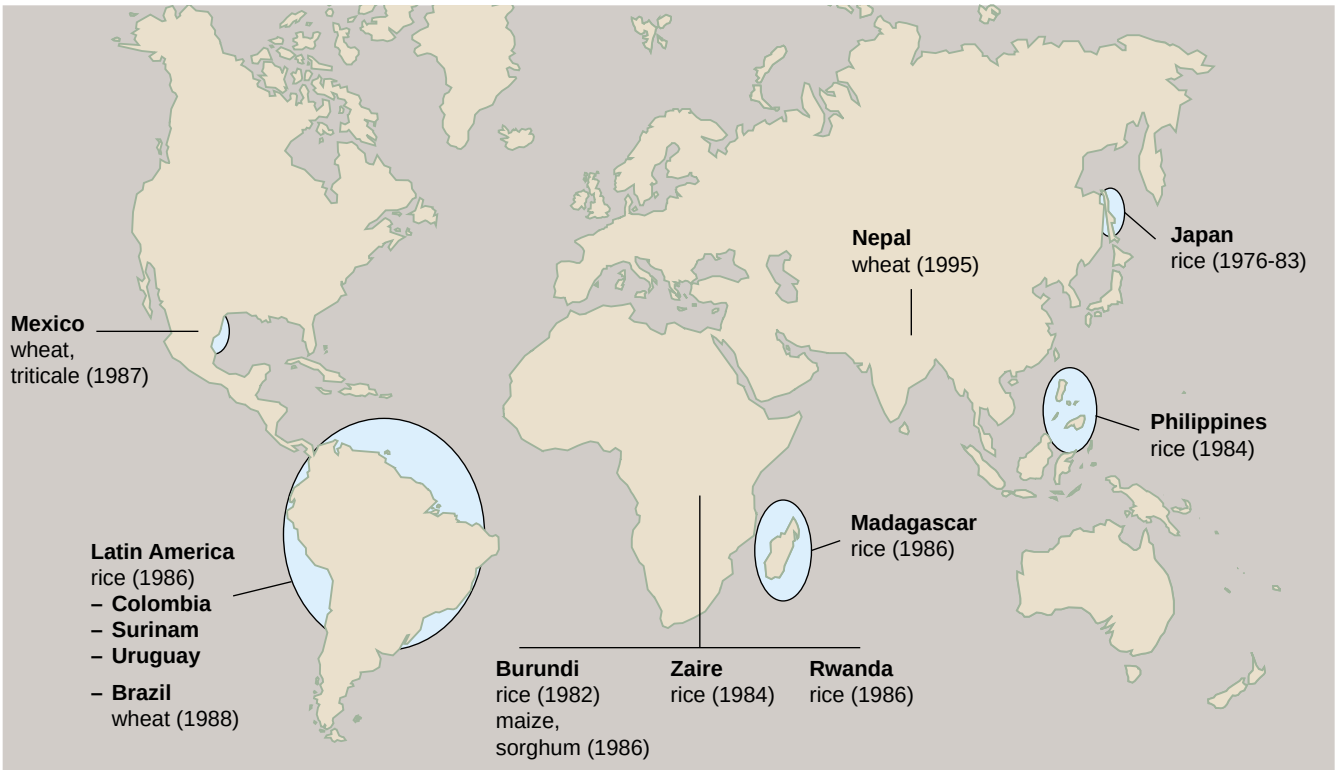
Bacterial sheath rot caused by *Pseudomonas fuscovaginae*

Bacterial brown sheath rot is caused by *Pseudomonas fuscovaginae* (ex Tanii, Miyajima and Akita 1976) Miyajima, Tanii, and Akita 1983 (Miyajima 1983; Miyajima *et al*. 1983). First reported on rice (Tanii *et al*. 1976), this pathogen has been found on other cereals, including wheat (Duveiller *et al*. 1989; Duveiller and Maraite 1990). Symptoms are characterized by irregular, angular, blackish brown lesions bordered by a purple-black water-soaked area. Infection often begins on the adaxial side of the sheath, where water is retained. The most severe cases result in poor spike emergence and sterility.

Pseudomonas fuscovaginae is a fluorescent pseudomonad, strictly aerobic, Kovacs' oxidase and arginine dihydrolase positive but negative for nitrate reduction and esculin hydrolysis. Pathogenic strains from wheat are similar to those isolated from rice with respect to non-production of 2-ketogluconate, acid production from trehalose but not from inositol, agglutination with antiserum against a reference strain, and pathogenicity on rice and wheat (Duveiller *et al*. 1988; Duveiller and Maraite 1990). These tests differentiate between *P. fuscovaginae* and other closely related non-pathogenic fluorescent pseudomonads that are Kovacs' oxidase and arginine dihydrolase positive. *Pseudomonas fuscovaginae* was found to produce syringotoxin, a toxic peptide (Pelsser *et al*. 1994; Flamand *et al*. 1996).

Little is known about environmental conditions conducive to bacterial sheath rot, but low temperatures and high humidity at the booting-heading stage are considered favorable. The disease has a broad geographical distribution (**Figure 4.3**), but its incidence on wheat and other crops is usually limited to highland locations. Up to now, the pathogen has been reported on wheat in Brazil (Malavolta *et al*.

Figure 4.3. Distribution of *Pseudomonas fuscovaginae* and year of identification on different host plants.



1988), Mexico (Duveiller and Maraite 1990) and Nepal (Anonymous 1995).

Dissemination through infected seed has been demonstrated (Duveiller and Martinez 1990) and, therefore, contaminated seed should be discarded before sowing. The pathogen can be identified from potentially infected seed by dilution plating on KBC agar (see Appendix).

No control measures are reported for wheat, except for the use of less susceptible genotypes and discarding infected seed. Little information is available on differences among genotypes regarding genetic resistance to this disease. Significant differences in percent spike exertion ranking from 44 to 77% are observed in diseased genotypes (Duveiller *et al*. 1991) (**Figure 4.4**). In Nepal, genotypes Annapurna-1, Annapurna-2, Annapurna-3 and WK685 have shown high levels of infection, but the disease was not observed on RR21 (Anonymous 1995).

Figure 4.4. Bacterial sheath rot of wheat caused by *Pseudomonas fuscovaginae* and reduced spike exertion.

Stem melanosis caused by *Pseudomonas cichorii*

Pseudomonas cichorii (Swingle) Stapp is a widely distributed pathogen that attacks numerous host plants (Bradbury 1986; Duveiller 1986). Its occurrence on wheat has been reported only in Canada, where the wheat cultivar Park appeared to be quite susceptible (Piening and MacPherson 1985).

Symptoms are first observed at the milky ripe stage, when small, light brown lesions develop beneath the lower two nodes. Later lesions darken and coalesce on the stem, rachis and peduncle, with occasional mottling on the glumes. The rachis, upper portion of the peduncle and portions of the stems immediately below the nodes turn dark. Heads are bleached and grain shriveled (**Figure 4.5 a and b**). The epidemiology of stem melanosis is not understood, but the combination of high humidity and high temperature conditions (29ºC) promotes its spread. Stem melanosis has been found to be associated with copper deficient soils (Piening *et al*. 1987; 1989).

The pathogen can easily be identified based on the following criteria. Tests are negative for arginine dihydrolase, levan and potato rot but positive for Kovacs' oxidase, fluorescence on King's medium B, nitrate reduction and tobacco hypersensitivity. The bacterium produces acid from glucose and trehalose but not from inositol and sorbitol.

There is no known method of control, but the application to copper-deficient soils of Cu chelate at 2-4 kg / ha Cu reduces stem melanosis and increases grain yield (Piening *et al*. 1987; 1989).

Other Diseases

Pink seed of wheat caused by *Erwinia rhapontici*

Pink seed caused by *Erwinia rhapontici* (Millard) Burkholder 1948 has been reported on wheat in Belgium (Dutrecq *et al*. 1990), Canada (Campbell 1958), England (Roberts 1974), France (Luisetti and Rapilly 1967), Russia and Ukraine (Diekmann and Putter 1995), and USA (McMullen *et al*. 1984; Forster and Bradbury 1990). Although it may affect both durum and bread wheat, this disease is considered insignificant. The pathogen should not be considered specific to wheat because hyacinth, onion, rhubarb and pea are also affected. It can be considered as an opportunistic pathogen that invades injured kernels (Wiese 1987).

Symptoms are distinct light pink kernels, slightly shrunken when compared to healthy grains (**Figure 4.6**). Pink seeds do not germinate well. Growth inhibition in wheat depends upon proferrosamine A concentration, with a threshold

Figure 4.5 a. Head melanosis in a field of Park wheat. b. Head melanosis on spikes of Traptow wheat. (Photos courtesy Lu Piening.)

Figure 4.6. Pink kernels on a wheat spike infected by *Erwinia rhapontici*. (Courtesy R.L. Forster.)

concentration in the range of host-unspecific toxins (about 10 ppm). Iron (Fe^{++}) uptake through plant roots is apparently inhibited via the iron chelating character of proferrosamines (Feistner *et al*. 1992; 1997).

The pathogen can be isolated on yeast dextrose carbonate agar (YDC) (see Appendix), in which it produces a pink diffusible pigment that can also be observed on peptone sucrose agar (PSA) (see Appendix). The bacterium is facultatively fermentative and tests positive for esculin hydrolysis, nitrate reduction and acid production from inositol. It gives negative results for oxidase.

Wheat spikes that are artificially inoculated with the pathogen develop a maroon color in the spikelet tissue surrounding the inoculation point (Diekmann and Putter 1995). Currently there are no known control measures.

White blotch caused by *Bacillus megaterium* pv. *cerealis*

Only one report describing white blotch on wheat has been recorded in the US (North Dakota) (Hosford 1982). The condition, considered a disease by Hosford (1982), is associated with *B. megaterium* de Bary 1884, pv. *cerealis* Hosford 1982. However, James G. Jordahl (personal communication) was not able to

repeat earlier results and concluded that *B. megaterium* pv. *cerealis* is not a wheat pathogen but rather an associated organism on leaves affected by physiological spotting. Therefore, white blotch syndrome should be carefully reviewed and the new results published.

Bacillus megaterium is a heterogeneous group that can be associated with soil, insects, plants (especially decaying) and fungi. It can survive extreme environmental conditions. However, the way in which wheat, *B. megaterium* pv. *cerealis* and the environment interact is relatively unexplored. The bacterium has been isolated from healthy leaves, which suggests that epiphytic growth is possible or that the organism is opportunistic, depending on the physiological stage of the plant, and probably not a true plant pathogen.

Smooth, white colonies on potato dextrose agar (PDA) (see Appendix) are characteristic of this Gram positive bacterium. Cells are non-motile, and average 3.5 µm in length and 1.8 µm in width. Ellipsoidal spores are produced that can be detected using the heat test described in Chapter 1. Acid, but not gas, is produced from glucose.

Conclusions

The purpose of this chapter was to make available to the reader information on diseases caused by bacterial pathogens that are largely unknown and not frequently observed on wheat. Epidemics caused by these bacteria are very sporadic and / or limited to ecological niches where favorable conditions may exist. Not much knowledge has accumulated on these pathogens, and the etiology and methods of control are largely unknown, except perhaps for spike blight caused by *C. tritici*.

The authors have summarized the literature available to clarify the importance of these wheat diseases. Their intention is to help workers who are unfamiliar with plant bacteriology to identify them. Since few studies have been done on these diseases, carefully conducting the appropriate determinative tests after confirming pathogenicity is highly recommended before reaching any conclusions. In certain cases it may also be advisable to contact a plant pathologist.

Appendix

General Purpose Media

All media should be autoclaved at 121ºC for 15 min, at 15 psi, and adjusted to pH 7.2 unless otherwise indicated.

Glucose yeast chalk agar (GYCA)
(Dye 1962; Schaad 1988)

Yeast extract	5.0 g
Glucose	5.0 g
Calcium carbonate (light powder)	40.0 g
Agar	15.0 g
Distilled water	1.0 L

This medium is useful for isolating *Clavibacter tritici* and storing strains. Cool the agar after autoclaving and mix well before pouring into plates so the calcium carbonate remains dispersed in the medium.

If tubes are prepared, they should be vortexed several times just before agar solidifies in order to obtain homogenous white agar slants. Care should be taken to use finely crushed calcium because coarsely crushed calcium will settle very fast, making it difficult to obtain homogenous slants.

GYS
(modified after Dye 1962)

Yeast extract	5.0 g
$NH_4H_2PO_4$	0.5 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	5.0 g
Glucose	5.0 g
Agar	12.0 g
Distilled water	1.0 L

Xanthomonads grow readily on this general purpose medium.

King's medium B (KB)
(King *et al*. 1954)

Proteose peptone (Difco No.3 / Oxoid L46)	20.0 g
K_2HPO_4	1.5 g
$MgSO_4 \cdot 7H_2O$	1.5 g
Agar	15.0 g
Glycerol	10.0 ml
Distilled water	1.0 L

This general purpose medium is particularly useful for detecting fluorescent pseudomonads, but other bacteria also grow easily on it.

Nutrient agar (NA)

Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled water	1.0 L

Prepared formulations of NA are commercially available and usually preferred. Nutrient broth is NA without agar.

Potato dextrose agar (PDA)
(Lelliott and Stead 1987)

Glucose	20.0 g
Potatoes (peeled and washed)	200.0 g
Agar	15.0 g
Tap water	1.0 L

This medium is not commonly used for culturing bacterial pathogens. It can be purchased as a prepared commercial formulation.

Peptone sucrose agar (PSA)
(Lelliott and Stead 1987)

Sucrose	10.0 g
Peptone	10.0 g
Agar	12.0 g
Distilled water	1.0 L

Adjust pH to 7.4.

Yeast extract-dextrose-calcium carbonate agar (YDC)
(Schaad 1988)

Yeast extract	10.0 g
Dextrose	20.0 g
Calcium carbonate (light powder)	20.0 g
Agar	15.0 g
Distilled water	1.0 L

Autoclave dextrose separately from the other ingredients. Cool the autoclaved medium to 50ºC and mix well before pouring the plates so the calcium carbonate remains suspended.

Xanthomonads produce large, yellow, domed, mucoid colonies on this medium, which can be used for general isolation from plant tissue.

Yeast peptone glucose agar (YPGA)
(Lelliott and Stead 1987)

Yeast extract	5.0 g
Proteose peptone	5.0 g
Glucose	10.0 g
Agar	12.0 g
Distilled water	1.0 L

Adjust pH to 6.5-7.0. Autoclave at 115ºC for 20 min.

Wilbrink's agar
(Sands *et al*. 1986)

Bacto peptone	5.00 g
Sucrose	10.00 g
K_2HPO_4	0.50 g
$MgSO_4 \cdot 7H_2O$	0.25 g
Na_2SO_3 (anhydrous)	0.05 g
Agar	15.00 g
Distilled water	1.00 L

Add:

Cycloheximide (in 2 ml of 75% ethanol)	75.00 mg

If cycloheximide is not available, it can be replaced by 250 mg benomyl.

Semi-Selective Media

CNS
(Gross and Vidaver 1979)

Bacto nutrient broth (Difco)	8.0 g
Yeast extract	2.0 g
Distilled water	1.0 L

After autoclaving, let cool to 50°C and add:

Nalidixic acid (solubilized in 0.1 M NaOH, 10 mg/ml)	25.0 mg
Polymyxin B sulfate (8000 USP units/mg fresh stock)	32.0 mg
Cycloheximide (10 mg/ml stock)	40.0 mg
Lithium chloride	10.0 g
Chlorothalonil (53% active, diluted 1:50 in sterile water)	0.0625 ml

Developed for isolating *Clavibacter nebraskensis* and useful for isolating *C. michiganensis* subsp. *tessellarius.* If Bacto nutrient broth is not available, substitute with nutrient broth (see under nutrient agar).

KBC
(Mohan and Schaad 1987, modified by Fessahaie 1993)

Proteose peptone (Difco No.3/Oxoid L46)	20.0 g
K_2HPO_4	1.5 g
$MgSO_4 \cdot 7H_2O$	1.5 g
Agar	16.0 g
Glycerol	10.0 ml
Distilled water	900.0 ml

Autoclave the solution and cool to 45ºC. Then add the following ingredients aseptically:

Boric acid (0.5% aqueous solution) autoclaved separately	100.0 ml
Cephalexin (stock solution of 10 mg/ml distilled water)	8.0 ml
Cycloheximide (stock solution of 25 mg/ml 75% methanol)	8.0 ml

KM-1
(Kim *et al*. 1982)

Lactose	10.0 g
D (+) trehalose	4.0 g
Thiobarbituric acid	0.2 g
K_2HPO_4	0.8 g
$KH_2PO_4 \cdot 7H_2O$	0.8 g
Yeast extract	1.0 g
NH_4Cl	1.0 g
Bacto agar (Difco)	15.0 g
Distilled water	1.0 L

Before adding the agar, dissolve the ingredients completely and adjust pH to 6.6 using 1 N NaOH. After autoclaving, cool the medium to 50°C. Then add:

Cycloheximide (dissolved in 95% ethanol)	100.0 mg
Ampicillin (dissolved in 50% ethanol)	1.0 mg
Tobramycin (dissolved in 50% ethanol)	8.0 mg

This medium has been proposed for isolating *Xanthomonas t.* pv. *translucens*.

Wilbrink's boric acid-cephalexin medium (WBC)
(Duveiller 1990b)

Bacto peptone	5.00	g
Sucrose	10.00	g
K_2HPO_4	0.50	g
$MgSO_4 \cdot 7H_2O$	0.25	g
Na_2SO_3 (anhydrous)	0.05	g
Agar	15.00	g
Distilled water	850.00	ml

Mix with the following solution (autoclaved separately):

Boric acid	0.75	g
Distilled water	150.00	ml

After cooling to 45ºC, add:

Cycloheximide (in 2 ml of 75% ethanol)	75.00	mg
Cephalexin (1 ml of a 10 mg / ml stock solution in 75% ethanol)	10.00	mg

Useful for isolating *X. t.* pv. *undulosa* and related pathovars.

XTS agar
(Schaad and Forster 1985)

Glucose	5.0	g
Nutrient agar (Difco)	23.0	g

After autoclaving and cooling to 45ºC, add:

Cycloheximide (20 ml of a 100 mg / ml stock solution in 75% ethanol)	200.0	mg
Cephalexin (1 ml of a 10 mg / ml stock solution in 75% ethanol)	10.0	mg
Gentamycin (0.8 ml of a 10 mg / ml stock solution in 75% ethanol)	8.0	mg

Developed for isolating *X. t.* pv. *translucens.* If commercial nutrient agar is not available, see recipe under nutrient agar.

Buffer

Phosphate-buffered saline (PBS)
(Lelliott and Stead 1987)

(0.01 mol / l pH 7.2)

$Na_2HPO_4 \cdot 12H_2O$	1.15	g
$Na_2HPO_4 \cdot 2H_2O$	0.40	g
NaCl	8.00	g
Distilled water	1.00	L

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Glossary

Abaxial—Situated on the side facing away from the axis or stem (dorsal); underside of leaves.

Adaxial—Situated on the side facing the axis (ventral); topside of leaves.

Aerobic—Relating to a microorganism that lives (or a process that occurs) in the presence of molecular oxygen; strictly aerobic means that it grows (or occurs) only under aerobic conditions.

Agar—A gelling material obtained from seaweed and used to prepare culture media on which microorganisms are grown.

Agglutination—A serological test in which bacteria or viruses suspended in a liquid collect into clumps when the suspension is treated with an antiserum containing specific antibodies for those particular bacteria or viruses.

Aggressiveness—A measure of the rate at which a virulent strain produces a given amount of disease; level of disease response to a pathogenic strain.

Anaerobic—Relating to a microorganism that lives (or a process that occurs) in the absence of molecular oxygen.

Antibiotic—A chemical compound that is produced by a microorganism and inhibits or kills other microorganisms.

Antibody—A new or altered protein (globulin) produced in a warm-blooded animal in response to an injected foreign antigen.

Antigen—Foreign proteins, and occasionally complex lipids and carbohydrates, which upon injection into an animal induce the production of antibodies.

Antiserum—Blood serum of a warm-blooded animal that contains antibodies.

Apex— Tip or pointed end of an organ.

Awn—One of the bristly fibers that terminate the head in some cereal grains.

Bacteria—The whole group of prokaryotic microorganisms. They are unicellular, multiply by fission, and lack chlorophyll and a true nucleus. Their DNA is usually contained in a single molecule.

Bactericide—A chemical compound that kills bacteria.

Bacteriophage—A virus that infects bacteria.

Biotype (biovar)—A subgroup within a species, usually distinguished by one or a few biochemical or physiological traits.

Booting—In cereals, the development of the flag leaf from whose sheath the inflorescence eventually emerges.

Carbohydrate—A compound made up of carbon, hydrogen and oxygen (CH_2O) with the last two in a 2:1 ratio.

Chlorosis—The yellowing of normally green tissue, due to chlorophyll destruction or lack of chlorophyll formation.

Coleoptile—The first leaf in the germination of monocotyledons that surrounds the succeeding leaves.

Colony—A group of individuals of the same species living in close association. A bacterial colony typically results from the multiplication of a single cell.

Cultivar—A released plant variety that has persisted under cultivation, as opposed to a botanical variety, which is a subdivision of a species.

Culture medium—Prepared food material on which microorganisms are grown.

Epidemic (epiphytotic)—A disease simultaneously affecting an unusually high number of individuals in a community or host population; widespread occurrence of a disease.

Epiphytic—Refers to organisms that grow and multiply on the surface of a plant without causing infection; however, some phytopathogenic bacteria may have an epiphytic phase in their life cycle.

Exudate—Liquid that contains bacteria and is discharged from diseased plant tissue.

Facultative anaerobe—An organism that is normally associated with aerobic conditions but also grows in fermentative (anaerobic) conditions.

Fermentation—Oxidation of certain substances in the absence of molecular oxygen.

Flag leaf—In cereals, the uppermost leaf from whose sheath the inflorescence emerges.

Funiculus—Small cord that attaches the ovule (seed) to the placenta.

Gene—Unit of inheritance that is located on the chromosome and determines or conditions one or more hereditary characters.

Genotype—Genetic makeup of an organism comprising the sum total of its genes, both dominant and recessive; a group of organisms (plants) with the same genetic makeup.

Germplasm—Genetic material that provides the physical basis of heredity; a collection of genotypes of an organism or plant.

Glumes—Chaffy or membranous two-ranked members of the inflorescence of grasses and similar plants, i.e., lower glume and upper glume, plus two sterile bracts at the base of a grass spikelet.

Guttation—Exudation of water from plants, particularly along the leaf margins.

Host—Living organism from which a parasite obtains its nutrients (e.g., wheat plant).

Hyaline—Colorless, transparent.

Hydathode—Structure at the tip of a leaf that allows guttation, a process in which water is discharged from the inside of the leaf to its surface.

Hypersensitive reaction—Host response to infection characterized by the rapid death of cells and tissues; this prevents further growth of the pathogen.

***In vitro*—**Outside the living body and in an artificial environment.

***In vivo*—**In the host, in the plant (*in planta*).

Indexing—A procedure that determines whether a given seed is infested by bacteria.

Infection—Establishment of a parasite within a host plant.

Infested—Refers to an environment (soil, seed, etc.) contaminated by bacteria.

Inoculation—The transfer of a pathogen onto a host.

Inoculum—Dispersal unit that contains a pathogen and is capable of initiating disease or is introduced for that purpose.

Isolation—A procedure in which a pathogen is separated from its host and cultured on a nutrient medium.

Lemma—The lower of the two glumes that surround each floret in the spikelet of the Gramineae.

Lesion—Discoloration of the host tissue around the pathogen's point of entry.

Levan—Fructose polymer produced by some bacteria.

Lipopolysaccharide (LPS)—Complex lipid structure in many Gram negative bacteria that contains unusual sugars and fatty acids, and constitutes the chemical structure of the outer membrane of the bacterial cell.

Mucoid—Appearance of bacterial colony producing slime.

Node—Point on the stem or branch at which a leaf or lateral is borne.

Oozing—Discharge or flux of bacteria that streams out of a cut made at the edge of a lesion and may be observed under the microscope.

Palea—The upper of two membranous bracts enclosing the flower in grasses.

Parasite—An organism that lives on or in another living organism (host) from which it obtains its food. It usually invades the host and causes disease.

Pathogen—An organism capable of causing disease.

Pathogenicity—Capability of a pathogen to cause disease.

Pathotype—Type strain that must accompany the description of a bacterium.

Pathovar—A strain or a set of strains with the same or similar characteristics, differentiated at the infrasubspecific level from other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more plant hosts.

Petri dish—Glass or plastic container consisting of a circular, flat dish with vertical sides, and a similar but slightly larger cover that fits over it; standard equipment for growing microorganisms in a pure culture.

Phenotypic—Relating to the external appearance of an organism as distinguished from its genetic constitution (genotypic).

Physiological races—Pathogens of the same species that are structurally similar, but different in their physiological and pathological characteristics.

Phytopathogenic—Parasitic organisms that invade living plants and cause a disease.

Plasmid—An extrachromosomal genetic element that is not essential for growth.

Pure culture—Culture resulting from a single bacterial colony.

Race—A genetically distinct group within a pathogenic species that infects a given set of plant varieties.

Rachis—Principal axis of an inflorescence or a compound leaf beyond the peduncle (petiole).

Resistance—The power of a host to overcome, completely or in some degree, the effect of a pathogen or damaging factor.

Saprophytic—Able to use dead organic material for food.

Seedling—Juvenile plant grown from the seed.

Serology—A method in which the specificity of the antigen-antibody reaction is used for detecting and identifying antigenic substances and the organisms that carry them.

Sheath—A tubular structure surrounding a plant organ or part, as for example, the lower part of the leaf clasping the stem in grasses.

Slant—Sterile agar medium congealed in test tubes in a slanting position.

Spore—A minute propagative unit that functions as a seed.

Stomata—Openings in the epidermis of a plant that are surrounded by guard cells.

Strain—The descendants of a single isolation in pure culture; a group of individuals of common origin; a pure culture obtained after sub-culturing a single colony.

Subspecies—Subdivision of a species based on minor but consistent phenotypic variations; genetically determined clusters of strains within the species.

Symptom—An external and internal reaction or alteration in a plant as a result of a disease.

Tiller—A shoot, culm or stalk arising from a crown bud in the Gramineae.

Translucent—Intermediate between clear and opaque, relative to light passage.

Type strain—One strain in a collection that is the reference specimen for the name of the species.

Uredospore—A binucleate, repeating spore of the rust fungi.

Virulence—The specific ability of a pathogen to overcome the host gene for resistance.

Water-soaked—Describes diseased tissues that appear wet, dark, greasy and, usually, sunken and translucent.

Whorl—Circle of leaves, flowers or roots arising from a single node or point.

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