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STUDIES ON BACTERIAL SPECK OF TOMATOES

CAUSED BY *PSEUDOMONAS SYRINGAE* PV *TOMATO*

A thesis presented in fulfillment of a
Masterate of Science by thesis only
at Massey University

by

Nicholas Brian Pyke

1980

ADDENDUM

Pg 1, Para 3: Agrobacterium tumefaciens - 'e' not 'a'

Pg 6, Para 1: feasibility - 'i' not 'a'

Pg 10, para 1: 1st sentence reads:
...oxidase reagant using the method of Kovacs (Lelliott et al 1966)

Pg 16, para 2: 'sera' not 'serum'

Pg 16, para 3: 'antiserum' not 'antisera'

Pg 21, para 2: distinguished - 'g' not 'q'

Pg 24, para 1: Klement (1963)

Pg 24, para 1: 24, 28 or 72 '48' not '28'

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Abstract

The taxonomy of the causal agent of bacterial speck of tomatoes is discussed and the trinomial *Pseudomonas syringae* pathovar *tomato* (Okabe) Young, Dye and Wilkie is adopted.

A vacuum infiltration method of artificially inoculating seed was used and *P.syringae* pv *tomato* was detected in both artificially and naturally infested seed using sensitive enrichment culture techniques. The pathogen can remain viable between seed harvest and sowing in association with seed but seed-plant transfer was only occasionally demonstrated.

The acid seed extraction method and other germicidal seed treatments were evaluated for their effect on the seedborne pathogen. Streptomycin sulphate as a slurry treatment (2.5g a.i./Kg of seed) just prior to seed sowing was the only totally effective seed treatment tested.

The potential for survival in infected crop debris, soil and on alternative hosts was shown. However, the pathogen was not isolated from weeds in infected tomato crops and no conclusive evidence of systemic infection was found.

1. INTRODUCTION

The tomato, *Lycopersicum esculentum* Mill., is an important fresh-market and processing crop in New Zealand. The North Island is responsible for 83% of the total production (Anon, 1977). A limited export market for fresh-market tomatoes, mainly to the Pacific Islands, also exists (Anon, 1978).

The area cultivated to tomatoes has been static for the last few years with approximately 800 hectares producing at an average of 68 tonnes per hectare (Anon, 1979). Processing tomatoes, one of the two major processing crops in New Zealand, occupy approximately two-thirds of the total hectarage in tomatoes but only produce about half the total annual yield of approximately 54,500 tonnes. Fresh-market tomatoes occupy the remaining area and produce the other half of the total yield.

This yield does not reflect the genetic potential of the crop due to the influence of a variety of adverse environmental factors, of which diseases frequently are an important part. A number of bacterial, fungal and viral pathogens infect tomatoes and often cause economic loss. One of the bacterial diseases, 'bacterial speck', caused by *Pseudomonas tomato* (Okabe) Alstatt, is regularly of economic importance causing significant yield losses (Tate and van der Mespel, 1976), usually in outdoor tomatoes but occasionally under glass. Other bacterial pathogens may also cause disease in tomatoes in New Zealand including:- *Agrobacterium tumefacians* Smith and Townsend causing crown gall; *Corynebacterium michiganense* (Smith) Jensen causing tomato canker; *Pseudomonas cichorii* (Swingle) Stapp causing stem bacteriosis; *Pseudomonas solanacearum* Smith causing bacterial wilt; *Pseudomonas syringae* van Hall causing stem necrosis; *Pseudomonas viridiflava* (Burkholder) Dowson causing internal stem rot and *Xanthomonas vesicatoria* Doidge causing bacterial spot.

Bacterial speck of tomatoes was first recorded in 1933 (Okabe 1933; Bryan 1933) and was first found in New Zealand, at Hastings, in 1944 (Reid, 1948) where it was causing serious stunting of a dwarf variety in the field and extensive leaf infection of seedlings in boxes. The disease is now widespread in New Zealand causing economic loss by way of reduced yields and delayed fruit maturity.

Yields are reduced when the tomato foliage is diseased (Schneider, Hall and Grogan, 1975; Grogan, Kimble, Schneider and Ioannou, 1974). The percentage yield loss varies with the plant growth stage at the time of infection; slight yield losses are to be expected even in diseased tomato seedlings which may recover when only the lower branches are diseased. The yield reduction in plants inoculated at the following stages:- (i) third true leaf; (ii) third true leaf and 50% flowering; (iii) third true leaf 50% flowering and first green fruit as compared to a healthy control, were 12, 13 and 13% respectively (Schneider, Hall and Grogan, 1975).

Grogan *et al* (1974) found significant yield reductions in all plants inoculated with the pathogen at seven weeks. Total yields in this trial were reduced by an average of 15% and total ripe fruit yield was reduced by 59%. The disease had no affect on the number of fruit set or on the average weight of either green or red fruit. However, there was a total yield loss due to the delayed maturity because ripe fruit weigh more than green fruit.

If plant roots are diseased the total or red fruit yield, as compared to a healthy control, is not significantly reduced. However, in diseased plants there is a delay of approximately six days to the 50% red stage (Grogan *et al* 1974). Top inoculation of 14 day old plants reduced total yield by 16% and red fruit yield by 20% while top and root inoculation of 14 day old plants reduced total yield and red fruit yield by only 11 and 13% respectively. The bacterium also had a synergistic effect on

maturity, delaying it by as much as 10 days. Root inoculation of 60 day old plants under glass reduced both root and stem weights by 38% and 26% respectively, although the general plant appearance was unaffected (Grogan *et al* 1974).

Although the disease may occur at any stage during the growing season, Schneider and Grogan (1977a) reported that infections occurring in the early spring caused the greatest economic loss. Pohronezny, Volin and Stall (1979) estimated a 50% cull rate of fruit harvested from diseased plots due to the small raised speck lesions which make the fruit unmarketable.

The disease is readily recognized by the characteristic symptoms on both fruit and foliage. The fruit lesions appear as small, dark, raised pustules on the fruit surface while foliar lesions are dark brown to black necrotic areas surrounded by yellow halos (Bryan 1933).

The species causing bacterial speck in tomatoes, *Pseudomonas tomato*, was erected by Okabe in 1933 and named *Phytomonas tomato* Okabe. In the same year Bryan (1933) described a bacterial pathogen of tomatoes and named it *Bacterium punctulans* Bryan. In Bergey's Manual of Determinative Bacteria (1939) *Bacterium punctulans* is listed as a probable synonym of *Phytomonas tomato*. Dowson in 1943, referred to the organism as *Pseudomonas punctulans* (Bryan) Dowson (Reid 1944). The name *Pseudomonas tomato* was proposed in 1944 (Alstatt 1944) and is now most commonly used in the nomenclature of the pathogen. However, recently Young, Dye, Panagopoulos, Bradbury and Robbs (1978) proposed a nomenclature and classification system for plant pathogenic bacteria in which *Pseudomonas tomato* is named *Pseudomonas syringae* pathovar *tomato* (Okabe) Young, Dye and Wilkie. Although the merits of this taxonomic system will be discussed subsequently, all pathogens shall, hereafter in this study, be referred to by the nomenclature of Young *et al* (1978).

Study Objectives

Although bacterial speck of tomatoes has the potential to cause considerable economic loss, the possible sources of primary inoculum have not been positively identified in New Zealand. This study aimed to identify and determine the importance of the possible sources of primary inoculum and investigate the effectiveness of different control measures. As conflicting opinions exist as to the importance of any seedborne spread of *Pseudomonas syringae* pv *tomato*, most of this dissertation deals with the possible importance of tomato seed as a source of primary inoculum. The importance of any other sources of inoculum was also investigated.

2. IDENTIFICATION AND TAXONOMY OF THE PATHOGEN

2.1 Introduction

Accurate pathogen identification is essential, even for routine work on a disease. Prior to identification, the pathogen has to be isolated by techniques which are sensitive and consistently enable the production of pure colonies. Several selective media have been developed to aid isolation of *Pseudomonas* species to pure culture (King, Ward and Raney, 1954; Kado and Heskett, 1970; Sands and Rovira 1970; Hildebrand, 1971; Pohronezny, Leben and Larsen, 1977).

While accuracy should be a prime criterion when identifying bacteria, methods should also be simple, rapid, and reliable. In the past, several different determinative schemes have been proposed for the identification of plant pathogenic pseudomonads (Lelliott, Billing and Hayward, 1966; Stanier, Palleroni and Doudoroff, 1966; Misaghi and Grogan, 1969; Sands, Schroth and Hilderbrand, 1970). However, as many of these techniques have proved to be inadequate, other techniques for the identification of *Pseudomonas* species have been developed. Techniques recognising the protein composition of the cell have been used by a number of researchers. Serological methods identifying the proteins (antigens), some of which are specific to a species, have been used to distinguish and identify *Pseudomonas* species (Lovrekovich, Klement and Dowson, 1963; Lucas and Grogan 1969; Taylor, 1970). Lester (1978) reported that gel electrophoresis, identifying the protein composition of the cell, appeared to be of value as a relatively simple and rapid method of distinguishing saprophytic and pathogenic *Pseudomonas* strains but is not sensitive enough to accurately identify species.

In this research, the scheme for identification as proposed by Lelliott *et al* (1966) was used and, because no determinative scheme available results in positive identification of *P.syringae* pv *tomato*, all tentative identifications were confirmed by pathogenicity testing on tomatoes. The feasability of using serological techniques to identify *P.syringae* pv *tomato* was investigated and the use of serology in some identification work is discussed. The nomenclature and classification of *P.syringae* pv *tomato*, as proposed by Young *et al* (1978), is considered and related to the determinative schemes and taxonomic approaches of a number of other researchers.

2.2 Morphological Characteristics of *P.syringae* pv *tomato*

P.syringae pv *tomato* is a gram negative, motile (1-7 polar flagella), aerobic, non-spore forming, non-acid fast rod shaped bacterium of approximately 1.3-2.3μ by 0.6μ (Bryan 1933; Wilkie & Dye 1974b). Although such a description may be useful it does not enable accurate identification as many closely related bacteria have similar properties.

2.3 Selective Media

It is of value to be able to grow a bacterial plant pathogen in pure culture to aid identification, pathogenicity and serological studies. Although *P.syringae* pv *tomato* will grow on a wide range of media the selectivity of these media varies considerably. Two growth media reported to show some selectivity for pseudomonads were used in this study.

The 'B' medium of King *et al* (1954, Appendix I) facilitates the production of two water soluble pigments, fluorescein and pyocyanin, the presence of which can be demonstrated by the fluorescence of cultures under ultraviolet light. Although contaminant micro-organisms may also grow on this media the selectivity can be increased by the addition of antibiotics which are ineffective against most fluorescent pseudomonads (Rovira and

Sands, 1970). Bashan, Okon and Henis (1978) added 2 µg of cyclohexamide and 200 units of penicillin per 10 ml of medium to prevent the growth of contaminant micro-organisms. In this study King's B medium was used for isolation to pure culture. The selectivity of the agar was increased by the addition of 20,000 units of penicillin per litre of medium, after the medium had been autoclaved and cooled to 45 C. In addition to the use of King's B medium agar to obtain pure cultures, on occasions the broth was used for primary isolations prior to subsequent transfer to King's B medium agar.

The D4 medium (Appendix I) of Kado and Heskitt (1970) shows some selectivity for pseudomonads, although some *Erwinia* species will also grow slowly on it. Schneider (pers. comm. 1979) reported that the selectivity of D4 broth for pseudomonads could be increased by the addition of 2-5g of Lithium chloride (Li Cl) per litre and the D4 agar could be made more differential by the addition of 1 ppm crystal violet.

P. syringae pv *tomato* colonies are slightly raised, circular and bluish-violet in colour on D4 agar plus crystal violet (Plate 1). Growth in D4 broth plus LiCl is slow with little colour change occuring. However, the liquids viscosity changes from the consistency of water to the consistency of a light oil.

D4 agar was used infrequently as it provided no advantage over King's B medium and all colonies on D4 had to be subcultured to King's B medium for determinative testing.

2.4 Determinative tests

Determinative tests can be an important aid in the accurate identification of phytopathogenic bacteria. Plant pathogenic pseudomonads have been organised into a number of groups on the basis of such tests. The number of groups and the division of the species into groups varies considerably with different researchers.

P. syringae pv *tomato* is usually grouped with other slow growing phytopathogenic pseudomonads (Lelliott *et al* 1966; Misaghi and Grogan 1969; Sands *et al* 1970; Doudoroff and Palleroni 1974).

Lelliott *et al* (1966) divided fluorescent pseudomonads into groups on the basis of twelve determinative tests. Using Lelliott *et al's* (1966) division of the pseudomonads it was possible to identify *P. syringae* pv *tomato* to group level by performing two simple tests, viz the oxidase and levan tests. These two tests enable *P. syringae* pv *tomato* to be differentiated from two of the three other fluorescent pseudomonads attacking tomato viz. *P. cichorii* and *P. viridiflava*. *Pseudomonas syringae* pv *syringae* and *P. syringae* pv *tomato* can not be differentiated by these two tests and in fact over 80% of the biochemical and nutritional tests performed by Misaghi and Grogan (1969) on these two pathovars gave the same result. Lelliott *et al* (1966) recorded some differences between *P. syringae* pv *syringae* and *P. syringae* pv *tomato* by use of 12 determinative tests, but these results varied with different researchers.

Although Lelliott *et al* (1966) consider fluorescence on King's B medium to be a variable criterion for most fluorescent pseudomonads, in this study on *P. syringae* pv *tomato* it was found to be a constant characteristic which together with the oxidase and levan tests, was regularly used to facilitate identification of *P. syringae* pv *tomato*.

Most pseudomonads fluoresce either green or blue on King's B medium under ultraviolet light in a darkened room. In this respect *P. syringae* pv *tomato* is no exception, always fluorescing blue under ultraviolet light 3500 Å (Plate 2). In natural light colonies appear light green in colour, slightly raised and rounded. (Wilkie and Dye 1974b) (Plate 3).

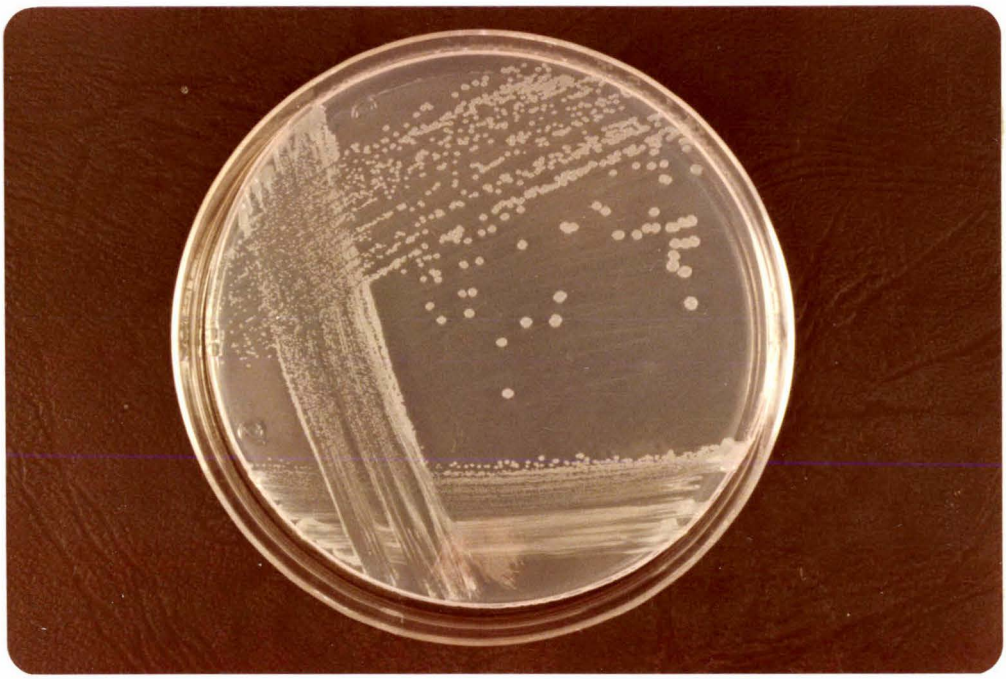


Plate 1: Appearance of *P. syringae* pv *tomato* colonies on D4 agar plus 1 ppm crystal violet.

The oxidase test is performed by streaking a loopful of growth, from a 24h nutrient or King's B medium agar culture, to blotting paper and covering it with oxidase reagant (Lelliott *et al* 1966). The apperance of a purple colour within ten seconds indicates a positive result. *Pseudomonas syringae* pv *tomato* was usually oxidase negative but a weak positive reaction sometimes resulted (Plate 4). Often fluorescence on King's B medium and the oxidase test were the only determinative tests used but the levan test was used on occasions.

A positive levan reaction, typical of *P. syringae* pv *tomato* (Plate 5) was white domed, mucoid colonies after 3 days growth on 5% sucrose agar (Lelliott *et al* 1966) (Appendix I).

Other determinative tests have been reported to aid the identification of *P. syringae* pv *tomato* but the variation in results obtained in different research laboratoriesmakes their value questionable.

2.5 Pathogenicity tests

Because *P. syringae* pv *tomato* can not be accurately separated from other closely related pseudomonads by determinative tests alone, accurate identification must involve the use of pathogenicity tests.

Pathogenicity tests were performed by inoculating tomato seedlings at the first or second true leaf stage with a 24-36h culture of the organism to be tested. A concentrated bacterial suspension was made using sterile water and this suspension was rubbed to the upper leaf surface using a cottonwool bud. Inoculated plants were incubated in isolation from other plants in a mist chamber at 25C.

This inoculation method was used because it was reported that infection was favoured by injury (Schneider and Grogan 1977b, Bashan *et al* 1978). Rubbing increases the number of injured and dead leaf trichomes through which the pathogen can infect the leaf

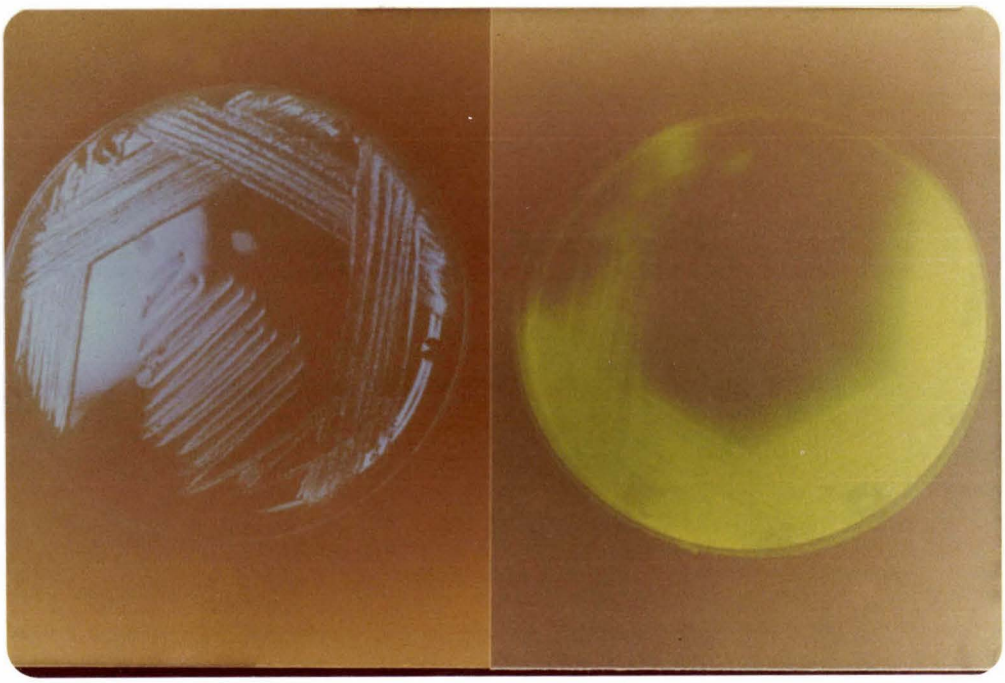


Plate 2: Fluorescence of bacterial colonies on King's B medium agar under ultraviolet light *left:* *P.syringae* pv *tomato;* *right:* green fluorescent *Pseudomonas* species.

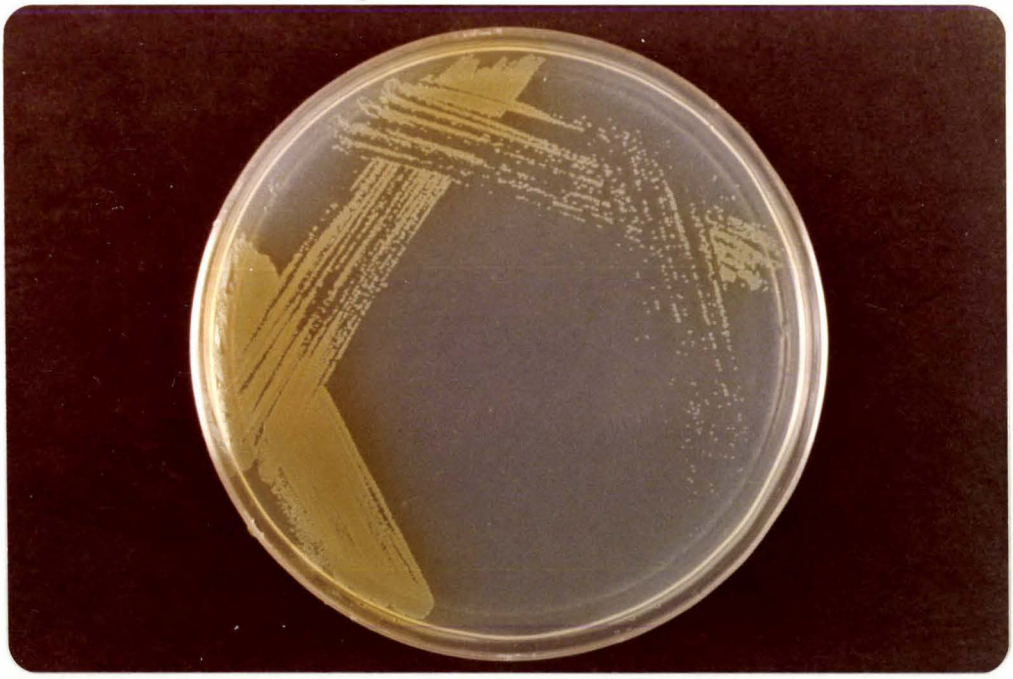


Plate 3: *P.syringae* pv *tomato* on King's B medium agar under natural light.

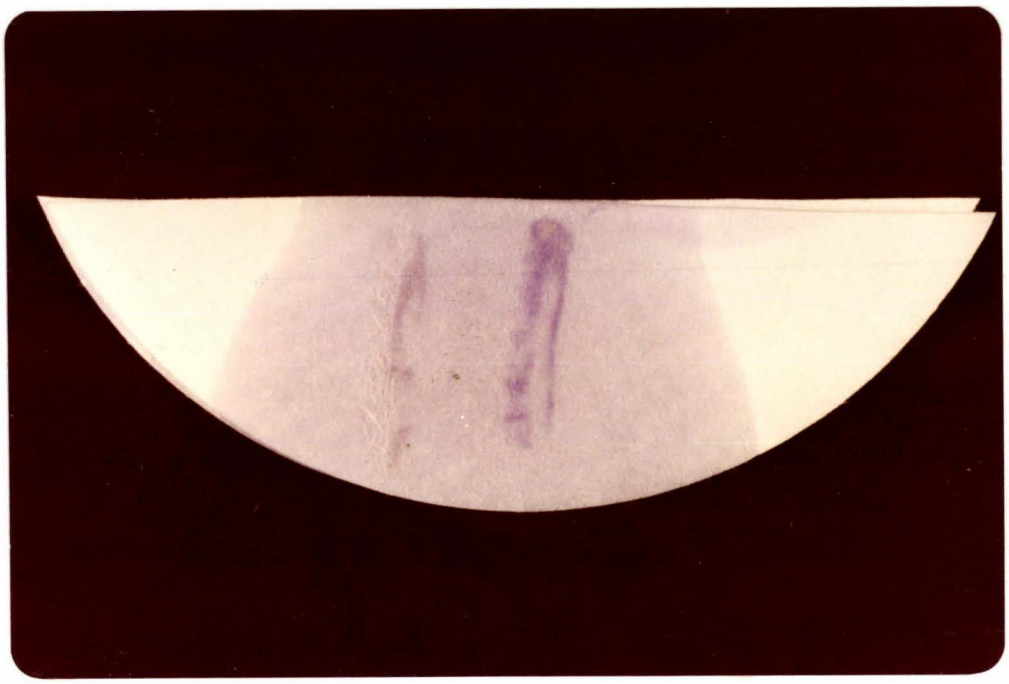


Plate 4: Oxidase reaction.
left: *P. syringae* pv *tomato* oxidase negative
right: *Pseudomonas* species oxidase positive

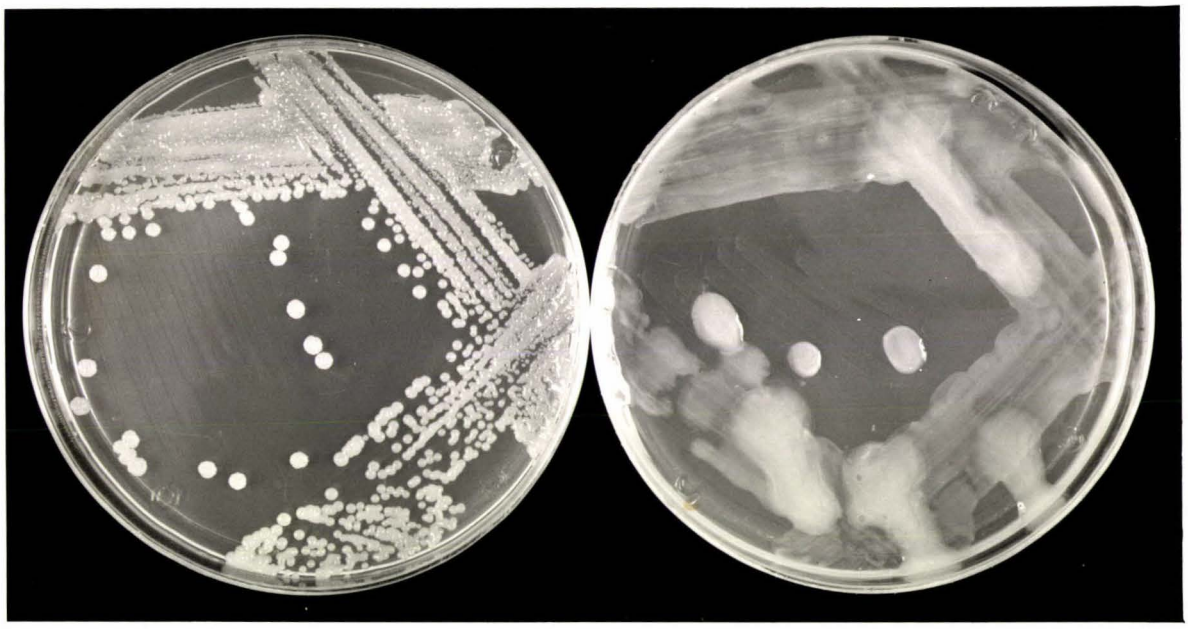


Plate 5: Appearance of bacterial colonies on 5% sucrose agar
left: *P. syringae* pv *tomato*, levan positive reaction
right: *Pseudomonas* species, levan negative reaction

tissue (Schneider and Grogan 1977b). Plants were incubated in a mist chamber to facilitate infection through the stomata (Schneider and Grogan 1977b, Bashan *et al* 1978). Basu (1966) and Grogan *et al* (1974) reported that disease development was favoured by 24-27C and 90-100% relative humidity. By incubating injured plants in a mist chamber at 25C it was hoped that disease development would be rapid and that low populations of the pathogen could be detected.

A positive result was recognised by the appearance of symptoms within 10 days, although recognisable foliage symptoms may appear after 4-5 days (Basu 1966).

Foliage lesions are initially irregularly shaped water soaked dark brown to black necrotic areas. Later they become dry, attain a more regular shape (2-3 mm in diameter) and are surrounded by yellow halos (Bryan 1933; Dye 1975) (Plate 6).

These symptoms are unlike those produced by other pseudomonads infecting tomato and are thus a useful guide to identification of the pathogen.

2.6 Serology

Serological tests can be used to accurately and rapidly identify plant pathogenic bacteria, providing the pathogen possesses some species specific antigens. Trigalet (1978) reported that serological cross reactions frequently occur between *Pseudomonas* species and can thus reduce the accuracy of the test. Taylor (1970) removed the non-specific heat-labile antigens of *Pseudomonas syringae* pv *phaseolicola* (Burkholder) Young Dye and Wilkie, which are common to 12 other plant pathogenic pseudomonads, by autoclaving the antigen or by absorbing non-specific antigens with a heterologous organism. This reduced non-specific cross reactions as the heat stable antigens of *P.syringae* pv *phaseolicola* are more specific and are shared by only two other

Plate 6: Tomato leaves artifically infected with *P.syringae* pv *tomato*

pseudomonads, viz: *Pseudomonas syringae* pv *morsprunorum* (Wormald) Young *et al* and *Pseudomonas syringae* pv *primulae* (Ark and Gardner) Young *et al*. The increased specificity of heat-stable pseudomonad antigens is also reported by other authors (Lovrekovich, *et al* 1963; Lucas and Grogan 1969).

Materials and Methods

Preparation of antisera to *P. syringae* pv *tomato* and *P. cichorii* isolates will facilitate accurate and rapid identification of the pathogens if a high-titred, species-specific antisera results from the preparation.

Antigen for injection was obtained by washing bacteria from the surface of several 48h, 25C nutrient agar cultures using 0.85% sterile saline. The concentration was adjusted to ca. 1.8×10^{10} cells/ml, estimated by use of the McFarland Scale (Barrett 1967).

Lucas and Grogan (1969) sonicated cells of *Pseudomonas syringae* pv *lachrymans* (Smith and Bryan) Young *et al*, prior to injection, to release more of the species-specific antigens which are usually associated with the cell wall. Schaad (1976) reported that the ribosomes of some *Xanthomonas* species show some specificity and may be useful in identification. Ribosomes can be released by sonication or in a French pressure cell.

In this study attempts to disrupt the cells by sonication for 15 mins. were unsuccessful. However, approximately 50% of the cells in the suspension were disrupted by use of a cold French pressure cell at 360 kg/mm^2. Preparations treated with a French pressure cell were stored at 4-5C until required for immunization.

One millilitre of disrupted cells was emulsified with 1 ml of Freund's Incomplete Adjuvant by repeated passage through a 10ml syringe. One millilitre of this emulsion was injected intramuscularly into each hind leg of a ca. 2kg rabbit. The intramuscular injection was repeated after 1 week and the final injection of 1 ml of adjuvant and 1 ml of antigen was made subcutaneously into 6 positions on the rabbit's back 2 weeks after the initial injection.

One week after the final injection 40 ml of blood was bled from the ear and the serum was separated from the clotted blood by decanting off after 1h, standing overnight at 4C, and centrifuging lightly. Pre-immune sera was collected prior to antigen injection.

The titre was determined by tube precipitin tests at 35C using approximately 10^6 whole cells/ml as antigen in 0.85% sterile saline. The titre of the antisera to *P.syringae* pv *tomato* was ca. 1:8.000 and the antisera to *P.cichorii* had a titre of ca. 1:5.000. Sera was stored frozen with 0.02% sodium azide as a preservative.

The antisera was used to test for antigen in gel double diffusion tests (Gibbs and Harrison 1976) in petri plates containing 0.4% phosphate buffered saline serology agar (PBSSA) (Appendix I). Circular well patterns were cut into the agar with a template and the antigen was added to the appropriate wells at approximately 10^6 cells/ml, either as whole cells or as heated (1 h at 100C) cells (Lucas and Grogan 1969). The antisera was diluted 1:4 with 0.85% sterile saline and added to the central well. Ten isolates of *P.syringae* pv *tomato* one *P.cichorii* isolate, and one unidentified *Pseudomonas* species isolated from the soil (Appendix III), were tested against *P.syringae* pv *tomato* antisera as both whole and heated cells. Three *P.syringae* pv *tomato* isolates, two *P.cichorii* isolates and the unknown *Pseudomonas* isolate were also tested against *P.cichorii* antisera as both whole and heated cell antigens. Pre-immune sera was used as a control.

Results and Discussion

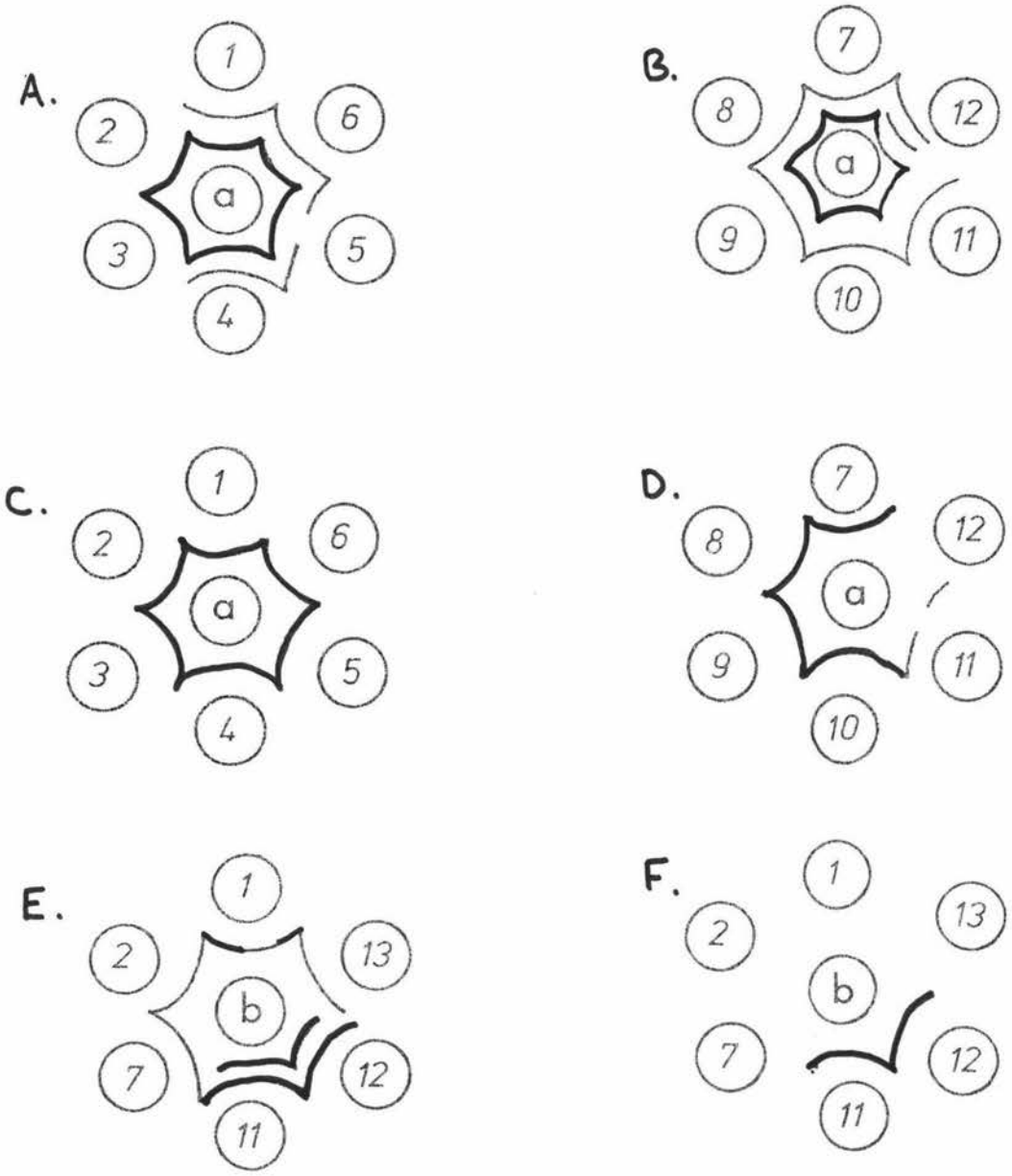
Using pre-immune sera no precipitation lines appeared in the control. The results obtained after incubation of the bacterial isolates and antisera for 5 days at room temperature are illustrated in Figure 1.

Non-specific reactions occurred when all whole cell preparations were reacted with the antisera. Although it is possible to differentiate between the different *Pseudomonas* species tested using whole cells, a more specific reaction was obtained when heated cells were used. The heat stable antigens were usually specific to the corresponding antisera, although a slight non-specific reaction occurred between the *P.cichorii* isolate and the antisera for *P.syringae* pv *tomato* (Fig. 1D). *P.cichorii* has been isolated from tomatoes in New Zealand but the disease is not as common as bacterial speck and the symptoms differ considerably (Dye 1975). However, some confusion in the use of serology to identify *P.syringae* pv *tomato* may develop as a result of a weak non-specific reaction of heat-stable antigens. It is possible that other closely related pseudomonads may also have non-specific heat-stable antigens which react with *P.syringae* pv *tomato* antisera.

Although antisera was only prepared to one isolate of *P.syringae* pv *tomato* it is possible that more than one serotype exists. These serotypes could differ in specific antigens and may not be accurately identifiable by using antisera produced to the one isolate tested, thus further limiting the usefulness of serological tests. This result was recorded by Lucas and Grogan (1969) when they established that 3 serotypes of *P.syringae* pv *lachrymans* existed, all differing in their specific antigens.

Figure 1

Gel double diffusion reactions of bacterial isolates against *P. syringae* pv *tomato* and *P. cichorii* antisera.



A and B Whole cell antigens of *P. syringae* pv *tomato* 1-10 in the corresponding wells, *P. cichorii* 1 in well 11 and, a *Pseudomonas* species I1 in well 12, against a 1:4 dilution of antisera (a) to *P. syringae* pv *tomato*.

C and D Heat stable antigens of:- *P. syringae* pv *tomato* cultures 1-10 in the corresponding wells, *P. cichorii* 1 in well 11 and, a *Pseudomonas* species. I1 in well 12 against a 1:4 dilution of antisera (a) to *P. syringae* pv *tomato*.

continued...

E. Whole cell antigens of: *P. syringae* pv *tomato* cultures 1,2 and 7 in the corresponding wells, *P. cichorii* cultures 1 and 2 in wells 11 and 12 respectively and a *Pseudomonas* species I1 in well 13, against a 1:4 dilution of antisera to *P. cichorii* (b)

F. Heat stable antigens of *P. syringae* pv *tomato* against a 1:4 dilution of antisera to *P. cichorii* (b).

If identification is to be based on serology it appears desirable to heat treat the pathogen to obtain a more specific reaction. As the pathogen had to be pre-cultured, prior to heat treatment, it was considered more useful in this study to use the pure culture for more time consuming but simpler, accurate and sensitive pathogenicity tests.

2.7 Taxonomy

In 1963 Lovrekovich, Klement and Dowson introduced a paper with the following paragraph: "Of the phytopathogenic pseudomonads the taxonomical position of the species *Pseudomonas syringae* van Hall is the most debated. Bacteria exhibiting a very wide host range belong to this species. Thus a number of supposedly new bacterial species have been described which have turned out to be representatives of *P. syringae*. There are indications that there are still bacteria denoted by separate names, which, however, probably also belong to the *Pseudomonas syringae* species".

Now, nearly twenty years later, the taxonomic position of *P. syringae* and related organisms is somewhat clearer but still much debated, despite a number of studies considering nomenclature and classification, having been made (Garber and Shaeffer 1963; Lovrekovich *et al* 1963; Lelliott *et al* 1966; Misaghi and Grogan 1969; Sands *et al* 1970; Dye *et al* 1974; Young *et al* 1978).

In this study the taxonomy of Young *et al* (1978) has been adopted because in their paper they reported "When the Approved Lists of Bacterial Names are published, to be effective from 1 January 1980, many commonly accepted names for bacterial pathogens will be discarded". The author of this thesis, considers this nomenclature and classification of the *P. syringae* group to be the most useful and accurate system currently available. Although this is the nomenclature and classification at present in vogue a better system may be proposed in the future thus rendering the nomenclature and classification herein obsolete.

A system of nomenclature and classification should:-

(i) accurately and unambiguously, yet simply, name and classify a pathogen and, preferably identify its pathogenic ability;

(ii) be used and accepted internationally;

(iii) be adaptable, accomodating new isolates or changes as further developments require;

(iv) classify by similarities.

The following comments are pertinent to these points:

(i) The out-dated system accurately and unambiguously named, classified and indicated the pathogenic ability of *P. syringae* pv *tomato* as *Pseudomonas tomato*. It did not always indicate this for other pseudomonads. The new trinomial nomenclature and classification does little to improve this although it usually does name, classify and indicate the pathogenic ability of the pathogen. In addition this system does invalidate many names which are either synonyms or unapproved.

(ii) The old binomial *Pseudomonas tomato* was generally accepted and used world-wide, although some authors preferred *Pseudomonas punctulans* (Bryan 1933; Neergaard 1977). The trinomial system is only likely to be implemented slowly.

(iii) The out-dated classification system was unable to readily accomodate new isolates, without proposing new species. This inflexibility of the system has been responsible for the accumulation of a large number of *Pseudomonas* species over the years.

Many pathogens were named twice and very closely related bacteria were classified as separate species. In the new system the number of *Pseudomonas* species has been reduced from over 220 (Dye *et al* 1975) to less than 70, including both species and pathovars (Young *et al* 1978). The infrasubspecific term, pathovar, implies some relationship with the type strain (Young *et al* 1978). New isolates can be classified as pathovars of

similar type strains and, if no similar type strain exists, be proposed as a new species.

(iv) The main difference between the old and new nomenclature and classification systems is the method by which pathogens are divided. In the out-dated system the bacteria were classified by differences. If a pathogen differed in any way from other species it was classified as a new species. In the new system classification is by similarities. Thus the pathogen is classified as a pathovar if a closely related type strain exists. Even using the old system the plant pathogenic pseudomonads were placed into groups by similarities in determinative tests. This grouping of the pseudomonads by similarity was used by various authors prior to the introduction of the Approved Lists of Bacterial Names on 1 January 1980 (Young *et al* 1978).

Bacterial species are usually distinquished by nutritional and biochemical tests, serological tests and pathogenicity tests. A number of researchers have worked on nutritional and biochemical tests for plant pathogenic pseudomonads, and accordingly *P.syringae* pv *tomato* appears in their lists.

Lelliott *et al* (1966) divided the fluorescent phytopathogenic pseudomonads into five groups, on the basis of 12 biochemical and nutritional (LOPAT) characteristics. These groups were differentiated primarily by the oxidase and levan reactions. *Pseudomonas syringae* pv *tomato* is found in LOPAT Group I, along with a number of other species from which it can not be distinguished by performing the twelve tests. All species in Group I have been reclassified as pathovars of *P.syringae* in the new nomenclature and classification system of Young *et al* 1978.

Misaghi and Grogan (1969) divided fluorescent plant pathogenic and saprophytic pseudomonads into two groups, with each group being further divided into two sub-groups on the basis of 86 nutritional

and biochemical tests. The division into groups I and II was made primarily on the arginine dihydrolase reaction. Sub-group IA included *P.syringae* pv *tomato* and other *Pseudomonas* species, all of which have been reclassified as pathovars of *Pseudomonas syringae* in the new classification and nomenclature system. Sub-group IB contains *P. cichorii* and *P. viridflava* because they differ in oxidase or levan reaction.

The percentage similarity was determined for all the isolates on the basis of the 86 nutritional and biochemical tests (Misaghi and Grogan 1969). The similarity of *P.syringae* pv *tomato* to other pathovars of the type strain is shown in Table 1.

Table 1: Percentage similarity of *P.syringae* pv *tomato* to other pathovars of the type strain on the basis of 86 nutritional and biochemical tests[a]. (Condensed and revised from Table 13 Misaghi & Grogan 1969).

	P.syringae pathovars						
	phaseo-licola	*glycinea*[b]	*lachrymans*	*syringae*	*sava-stanoi*[c]	*tabaci*[d]	*mori*[e]
P.syringae pv *tomato*	81	77	75	81	78	75	83

[a]Each figure at the intersection of two pathovars is indicative of their percentage similarity. The higher the percentage the greater the similarity

[b]*Pseudomonas syringae* pv *glycinea* (Coerper) Young, Dye & Wilkie

[c]*Pseudomonas syringae* pv *savastanoi* (Smith) Young *et al*

[d]*Pseudomonas syringae* pv *tabaci* (Wolf and Foster) Young *et al*

[e]*Pseudomonas syringae* pv *mori* (Boyer and Lambert) Young *et al*

Sands *et al* (1970 classified *P. syringae* pv *tomato* and a large number of phytopathogenic pseudomonad isolates into Group I by their slow growth rates or by the arginine dihydrolase test. Group I isolates were all oxidase negative, with the exception of *P. cichorii*, and have since been reclassified as pathovars of *P. syringae* (Young *et al* 1978). The Group I phytopathogenic pseudomonads were divided into sub-groups, on the basis of 188 tests. Although each sub-group corresponded with a species, some sub-groups contained more than one species due to similarities in test results (Sands *et al* 1970).

The inability of Lelliott *et al* (1966) and Sands *et al* (1970) to distinguish between species in Group I, by the use of nutritional or biochemical tests, and the demonstration of a high percentage similarity between species of Group I by Misaghi and Grogan (1969) indicates that all these 'separate species' are often indistinguishable by use of nutritional and biochemical tests. Furthermore they would be more accurately classified as pathovars of *Pseudomonas syringae*.

Nutritional and biochemical tests are not the only way pseudomonads can be identified. Serology and phage sensitivity tests are often very sensitive and specific methods providing useful information on the identity and relationship of bacteria. As mentioned earlier, heat-stable antigens are more specific than heat-labile antigens in serological tests, although some non-specific reactions will occur. Twelve percent of the serological reactions of antigens with the antisera of *Pseudomonas syringae* pv *pisi* (Sackett) Young *et al* were non-specific while only one percent of the phage tests were non-specific (Taylor 1972). Lovrekovich *et al* (1963) reported that some strains of *P. syringae* pv *syringae* have no common antigens while *Pseudomonas syringae* pv *morsprunorum* (Wormald) Young *et al* and *P. syringae* pv *syringae* were often indistinguishable using either serological, nutritional, or biochemical tests. *Pseudomonas syringae* pathovars can be identified by gel double diffusion tests but the range of the serological variability of each pathovar needs to be determined before it can be used with confidence (Lucas and Grogan 1969).

Klement (193) reported that because of the similarity of biochemical properties, colony morphology, antigenic structure and phage sensitivity tests, pathogenicity testing is the sole reliable method of identifying plant pathogenic pseudomonads. From the above discussion it is obvious that *P. syringae* pv *tomato* can not be accurately identified by determinative tests alone. It is also probable that any identification made using serology or phage sensitivity would not be entirely accurate. Thus, at present, the only way an accurate identification can be made is by way of pathogenicity tests. If new determinative tests are developed which express different relationships then these may, not only aid identification, but could also result in a change in nomenclature and classification of fluorescent pseudomonads.

3. SEED - A SOURCE OF PRIMARY INOCULUM?

3.1 Introduction

Because tomatoes are a relatively short term crop it is important that the pathogen remains in a viable state between crops so that it can act as primary inoculum in the new crop. Tomato seed may provide such a source of primary inoculum.

P. syringae pv *tomato* was first reported to be seedborne by Bryan (1933), who observed dark lesions on the cotyledons of seedlings soon after emergence when 'inoculated' seed was planted in clean soil. Alstatt (1944) associated the high percentage of plants infected with bacterial speck with the fact that the grower did not 'treat' his seed. Although Reid (1948) does not state that the disease is seedborne, his terminology implies that the high percentage of seedling infection observed was due to the use of imported, infested seed. Basu (1966) recognized that bacterial speck was causing problems in nursery situations but did not suggest what the source of primary inoculum was.

Grogan *et al* (1974) developed enrichment culture techniques enabling detection of 1 artificially inoculated seed in 10,000, but this technique gave negative results in attempts to isolate *P. syringae* pv *tomato* from eight commercial seedlines suspected of harboring the pathogen. Bacterial speck was not detected on any plants grown from artificially infested seeds, even when plants were incubated under environmental conditions believed to favour speck development. However, the pathogen could be recovered from the seed. Grogan *et al* (1974) concluded that although *P. syringae* pv *tomato* may be present in commercial seedlots, it is probably not the primary source of inoculum for speck epidemics; therefore, seedborne inoculum is probably not important in the year to year occurence of bacterial speck. However, they do suggest that the organism may be initially introduced into a new area in association with the seed,

and that this could then be followed by a population increase prior to the appearance of a speck epidemic.

Chambers and Merriman (1975) isolated *P. syringae* pv *tomato* from seed, hand extracted from tomato fruits infected with bacterial speck. They were unable to isolate the speck organism from seed extracted from the fruit by either acid or fermentation processes, the two methods most regularly used to extract tomato seed.

Although there was no conclusive evidence available which associates the occurence of speck with the pathogen being seedborne, many researchers believed the pathogen to be seedborne. Tate and van der Mespel (1976) reported that the bacterium overwintered in seedlines and these contaminated seedlines resulted in seedling infection in the nursery but no data is available to support their claims.

Schneider and Grogan (1977a) reported the sources of inoculum for the sporadic epidemics in processing tomatoes in California had not been identified, but attempts to demonstrate the presence of the bacteria in seedlines, in earlier unpublished work, were unsuccessful. They went on to suggest the speck organism was soilborne or ubiquitous. Neergaard (1977) in a survey of seedborne bacteria listed *P. punctulans* (a synonym of *P. syringae* pv *tomato*), citing the work of Reid (1948). Reid's work did not justify the conclusion that the pathogen was seedborne.

The lack of any conclusive evidence that *P. syringae* pv *tomato* is seedborne, or that other sources of primary inoculum exist in New Zealand, demonstrated the need for work to identify the sources of primary inoculum.

While this research was in progress Bashan *et al* (1978), in Israel, detected the speck organism in 2 out of 3 commercial seedlines tested. Infected seedlings were regularly obtained from the germination of these two seedlines whereas those grown from clean seed, under the

same conditions, were disease free. These researchers believe the bacteria are surface contaminants as they were unable to isolate the pathogen from seeds, or the resultant seedlings when the seed was 'surface sterilised' using an infiltration technique. More recently Kim (1979) reported that seeds carrying *P.syringae* pv *tomato* produced plants with leaf symptoms in one month at 17-26C in a moist chamber.

Much of this study has concentrated on trying to establish the possible importance of seedborne spread of *P.syringae* pv *tomato* in New Zealand. The association of any pathogen with the seed is important for the following reasons:

1. It allows the pathogen to remain in constant association with a susceptible host.
2. It enables the pathogen to survive in the absence of susceptible host plants.
3. It enables the widespread dispersal of the pathogen.

Many bacterial plant pathogens are seedborne including many of the fluorescent pseudomonads which are closely related to *P.syringae* pv *tomato* viz: *P.syringae* pv *syringae*, *P.syringae* pv *tabaci* *P.syringae* pv *phaseolicola*. It seems logical to postulate that *P.syringae* pv *tomato* could also be seedborne. If the speck organism was seedborne this could explain the widespread distribution and regular incidence of the disease, both in the nursery and in the field.

There are 3 requirements for a pathogen to be seedborne:

1. Transfer from the plant to the seed i.e. either contamination or infection of the seed.
2. Survival of the pathogen in association with the seed between harvest and sowing. This step may require the pathogen to survive some seed treatments.
3. The infection of either seedlings or older plants via inoculum associated with the seed.

The possible seedborne spread of the *P.syringae* pv *tomato* is considered with regard to these 3 requirements.

3.2 Plant-Seed Transfer

This study only aims to establish that plant-seed transfer can occur in a natural situation, not how the pathogen becomes associated with the seed. Prior to establishing that *P.syringae* pv *tomato* can be associated with the seed it was necessary to develop techniques for isolating the pathogen from seed and identifying it, either while still in association with the seed or following isolation from the seed. All isolation and identification techniques were tested for sensitivity, accuracy and usefulness utilising artificially inoculated seed.

3.2.1 Inoculation of Seed

Bryan (1933) reported that infected seedlings were obtained when seed inoculated with *P.syringae* pv *tomato* was planted in clean soil. There was no reference to the method used to inoculate seed. Grogan *et al* (1974) used artificially contaminated seed in attempts to demonstrate seedborne spread and to artificially infest seedlines but again the method of contamination was not reported.

In this study two techniques were successfully used to artificially inoculate seed and a further two techniques, intended to more closely simulate how the pathogen could become associated with the seed in a natural situation, were unsuccessful.

(a) Artificial contamination

Materials and Methods

The seed of five tomato cultivars 'Castlong', 'Dorchester', 'Scoresby', 'UC134' and 'VF 145-B-7879' (the origins of all seedlines used in this study are shown in Appendix II) were artificially

contaminated with *P. syringae* pv *tomato* in the following manner:

A loop full of a virulent strain of the pathogen was added to 14 ml of King's B medium broth (King *et al* 1954) in a wide neck McCartney bottle and incubated at 25C for 24 h. Two 4g samples of surface sterilised (70% ethanol for 10 minutes) tomato seed of each variety were added to the broth culture and incubated at 25C for either 24, 28 or 72 h. Three replicates of each variety were made for each time interval. At the appropriate time seed was removed from the broth and dried on blotting paper at room temperature. Following incubation some seed from each replicate was surface sterilised in an attempt to establish the nature of the association of the pathogen with the seed. Isolations were made from all replicates to determine the most appropriate incubation interval, the effect of surface sterilisation, and the susceptibility of different varieties to artificial contamination.

Results and Discussion

The pathogen was isolated from 'Castlong', 'Dorchester', 'UC 134' and 'VF 145-B-7879' but not from 'Scoresby' seed (Table 2).

Table 2: The effect of inoculation treatment on the artificial contamination of tomato seed

	Inoculation Treatment (hours incubation)					
Variety	24	48	72	24+ Surface ster.	48+ Surface ster.	72+ Surface ster.
'Dorchester'	+	+	+	-	+	+
'Castlong'	+	+	-	-	-	-
'VF 145-B-7879'	+	+	-	-	-	-
'UC 134'	+	-	-	-	-	-
'Scoresby'	-	-	-	-	-	-

\+ = *P. syringae* pv *tomato* was recovered from seed

\- = *P. syringae* pv *tomato* was not recovered from seed

The 'Scoresby' seed was treated with the fungicide 'Spergon^{R} and although the seed was thoroughly rinsed prior to inoculation it is possible the Spergon^{R} treatment had a bactericidal effect (see seed treatments), thus explaining why the pathogen was not recovered from this seedline.

The pathogen was recovered from the other 4 seedlines after 24 h but not always after longer incubation periods. More contaminant bacterial colonies were evident in the isolates from long incubation periods than from short incubation times. The short incubation times probably did not allow the contaminant bacteria time to establish and compete with the pathogen. When longer incubation periods were used any contaminants present probably competed more successfully with the pathogenic isolate. Contaminants probably became associated with the seed when it was dried following surface sterilisation.

The pathogen was recovered from 5% or 2 of the 36 isolations made from 'Dorchester', 'Castlong', 'VF 145-B-7879' and 'UC 134' seed which was surface sterilised following inoculation. This indicates that the pathogen was usually associated with the seed in such a way that surface sterilisation inactivated the bacteria.

The pathogen was most consistently recovered from 'Dorchester' and 'VF 145-B-7879' seed so all further artificial contamination work was done by incubating these varieties in a 24 h King's B medium broth culture of the pathogen, at 25 C for 24 h.

(b) Vacuum infiltration

A rapid inoculation method, resulting in a high percentage of seed becoming infected or contaminated, would be of greater benefit in future study than the artificial contamination method. Schaad and Kendrick (1975) artificially inoculated crucifer seed with *Xanthomonas campestris* pv *campestris* (Pammel) Dowson by a vacuum infiltration process. The seed to be infiltrated was secured to the base of a petri plate with double stick tape and 1 µl of the pathogen suspension was vacuum infiltrated through a small hole made in the seed coat.

Materials and Methods

In this study the technique of Schaad and Kendrick (1975) was modified to enable rapid inoculation of large numbers of seed. Initially seed to be vacuum infiltrated was injured by placing a small hole in the seed coat with the tip of a sterile needle. Later this injury process was dispensed with as it was extremely time consuming and had a detrimental effect on seed germination. The seed to be inoculated and a concentrated bacterial suspension of *P. syringae* pv *tomato* were put in a McCartney bottle. The bacterial suspension was prepared by flooding a 24 h agar culture of the pathogen with sterile water. The bacterial growth was scraped off with a sterile loop and the resultant suspension was added to the McCartney bottle at the rate of 1 µl/seed or 0.5ml/g as there are approximately 500 seeds/g.

The seed-bacterial suspension mixture was placed under vacuum for 2-3h. Further drying of the seed on blotting paper, at room temperature or in a 16C refrigerated incubator, was usually necessary. Results obtained with this inoculation process were determined, by use of the Cookes microtitre plate system described in Section 3.2.2.

The percentage of infested seed in the five inoculated seedlines, 'Dorchester', 'Fireball', 'UC 134', 'VF 145-B-7879' and 'Scoresby' was determined following vacuum infiltration. The number of seeds tested in each seedline varied according to the availability of the seedline and the success of the infiltration process.

Results and Discussion

The percentage of seed from which the pathogen could be recovered varied with the different seedlines (Table 3). Although it was expected that the pathogen would be recovered less regularly from uninjured seed than from injured seed this was not the case (Table 4).

Table 3: Recovery of *P. syringae* pv *tomato* from five vacuum infiltrated seedlines

Variety	Total number of seeds tested	Total number of seeds from which pathogen was re-isolated	Percentage of seeds from which the pathogen was re-isolated
'Dorchester'	1056	219	20.7
'Fireball'	288	3	1.0
'UC 134'	192	83	43.3
'VF 145-B-7879'	1152	646	56.0
'Scoresby'	384	0	0

Table 4: Recovery of *P. syringae* pv *tomato* from injured and uninjured vacuum infiltrated 'VF 145-B-7879' seed

Treatment	Total number of seeds tested	Total number of seeds from which pathogen was re-isolated	Percentage of seeds from which pathogen was re-isolated
injured seed	960	527	54.8
uninjured seed	192	119	62.0

Vacuum infiltration resulted in high percentage infestations of 'Dorchester', 'UC 134' and 'VF 145-B-7879' seedlines. 'Fireball' seed was very difficult to vacuum infiltrate and all attempts to vacuum infiltrate 'Scoresby' seed, which had been treated with the fungicide Spergon^{R}, were unsuccessful.

Spergon^{R} may have bactericidal activity, thus explaining the difficulty encountered in all attempts to vacuum infiltrate this seedline. The 'Fireball' seed had not been treated so the difficulty encountered in all attempts to vacuum infiltrate this seedline can

not be readily explained. 'Dorchester' and 'VF 145-B-7879' seedlines were used in most subsequent work because high percentage infestations resulted from vacuum infiltration and both seedlines were readily available. Although a high percentage infestation resulted with vacuum infiltrated 'UC 134' the supply of this seedline was limited. Contrary to expectations, injury did not appear to increase the success of the vacuum infiltration process. In this trial a higher percentage of infested seed resulted when the seedcoat was not injured justifying the omission of the time consuming injury process from the vacuum infiltration process.

(c) Fruit Inoculation

In recent years some attempts have been made to establish whether *P.syringae* pv *tomato* can systemically infect tomatoes Grogan *et al* (1974) reported that although the pathogen could be recovered from necrotic spots on the roots it did not invade the plant systemically. Bashan *et al* (1978) also concluded that bacterial speck was a local-lesion, rather than a systemic disease. If this is correct then the only way the pathogen can infest the seed is from infected fruit or foliage. In this work attempts were made to obtain natural association of the pathogen with the seed by fruit inoculation.

Bryan (1933) reported that speck lesions developed when young fruit were swabbed with a water suspension of the speck organism or when the fruit were thrashed with a leaf and sprayed with the suspensions. Both these inoculation methods injure the fruit hairs through which the bacteria are presumed to penetrate.

Materials and Methods

In this study disease-free 'Moneymaker' fruit were picked at the early green stage and inoculated with a virulent isolate of *P.syringae* pv *tomato* by swabbing with a cotton wool bud. Inoculated fruit were placed under high relative humidity at 25C and observed

for lesion production.

Mature seeds can not be extracted from immature fruit so attempts were made to inoculate disease-free maturing 'Moneymaker' fruit in order to extract mature seed. Ten fruit, varying in stage of maturity from middle green to early red, were collected from a glasshouse crop, showing no evidence of bacterial speck, and each sample was inoculated in one of the following ways:

(i) A concentrated suspension of *P.syringae* pv *tomato* was carefully injected under the fruit's epidermis by use of a sterile syringe and a sterile 25 gauge hypodermic needle. Fruit were injected with sterile water as controls.

(ii) The fruit were injured by scratching with a sterile needle and a concentrated suspension of the pathogen was rubbed onto the fruit with a cotton wool bud. Controls were scratched and inoculated with sterile water.

(iii) A concentrated suspension of the pathogen containing celite was rubbed onto the fruit with a cotton wool bud. Controls were inoculated with a suspension of sterile water and the abrasive in the same manner.

(iv) A concentrated pathogen suspension was rubbed onto the fruit using a cotton wool bud. Controls were similarly treated with sterile water.

All fruit were incubated at 25C at a high relative humidity by placing them in sealed plastic containers containing damp blotters. Controls were placed in separate containers.

Young, early red stage disease-free 'Moneymaker' fruit were also inoculated while still attached to the plant in the same manner as above. Following inoculation each fruit was wrapped in a plastic bag, to prevent disease spread to other plant parts and

to create a high relative humidity favouring disease development. Inoculated fruit were kept until maturity or until isolations for *P. syringae* pv *tomato* were made.

Results and Discussion

Only a few bacterial speck lesions, small, dark round pustules usually 1-2mm in diameter (Plate 7 & 8) (Bryan 1933), (Dye, 1975), appeared on 'Moneymaker' fruit inoculated at the early green stage in the laboratory, after 7-10 days incubation. These lesions were readily removed from the fruit with a sterile needle, ground in sterile water and streaked to King's B medium. After 36 h at 25 C colonies typical of *P. syringae* pv *tomato* developed on culture plates. Subsequent pathogenicity tests were positive but because the fruit were immature no mature seeds were extracted.

No lesions typical of bacterial speck developed on any of the mature fruit inoculated in the laboratory. However, the pathogen was isolated from fruit, inoculated by injecting the bacterial suspension under the epidermis, both 2 and 4 weeks after inoculation. The failure of the pathogen to cause symptom production in older fruit is in keeping with the findings of Bryan (1933).

No fruit lesions developed on any of the fruit inoculated in the glasshouse, but foliar lesions developed on any sepals leaves or petioles enclosed in the bag. The pathogen was readily isolated from all foliar lesions and all fruit inoculated with the pathogen after both 3 and 6 weeks. The pathogen was not isolated from any of the controls.

As symptom production only occurred on fruit detached from the plant and inoculated at the early green stage it was expected that symptoms would also develop on fruit inoculated at the early green stage on the plant. However, this did not occur because sepals and petioles were so susceptible to infection that premature fruit

Plate 7: Bacterial speck lesions on naturally infected tomato fruits .

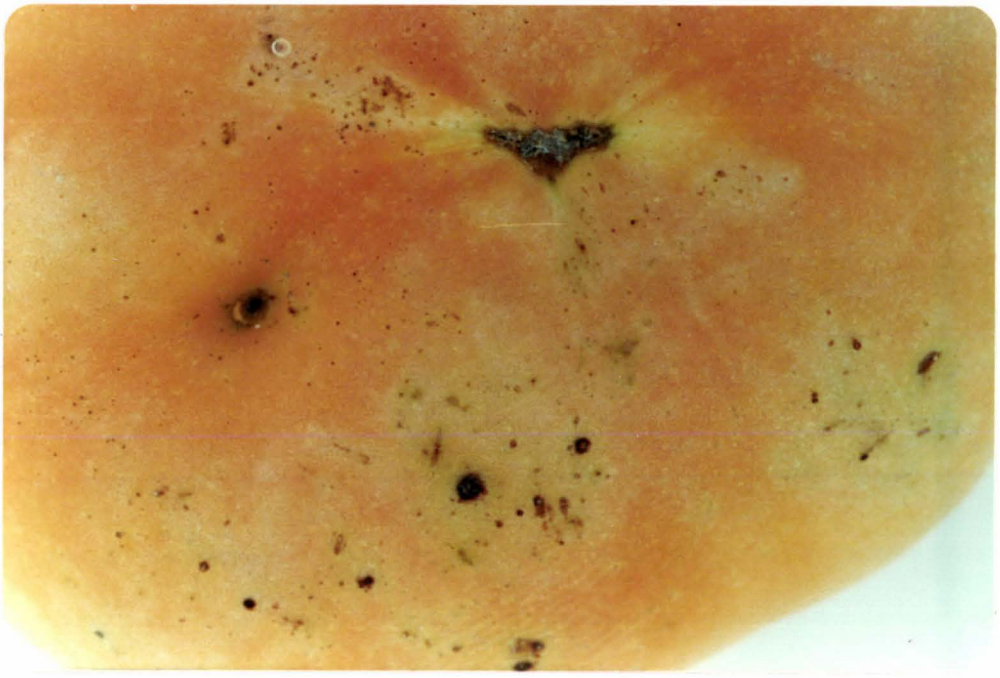


Plate 8: Delayed maturity around the bacterial speck lesions on a naturally infected tomato fruit.

drop and rot occurred in the artificial incubation conditions. Unfortunately it was not possible to incubate inoculated fruit without covering them as it was necessary to prevent spread of the disease to other fruits in the commercial glasshouse crop.

It had been hoped that artificial inoculation would result in a large number of infected fruits with a high percentage of each individual fruit being infected. Had a high percentage infection resulted then possibly some of the seeds harvested from the fruit may have been infested enabling the use of naturally inoculated seed in subsequent work.

(d) Natural inoculation

Although a high percentage of seeds can be infected by artificial inoculation methods, a method simulating what may occur in nature would be advantageous in this work. Because attempts to obtain seed infestation by inoculating the fruit were largely unsuccessful the following seed inoculation method was tried.

Materials and Methods

One gram of leaf material, with lesions typical of *P. syringae* pv *tomato*, was placed in a McCartney bottle containing 2 ml of sterile water. A gram of 'VF 145-B-7879' seed was added to each bottle and incubated for 48 hours at 25C. Seed was separated from leaf tissue, dried and attempts were made to isolate the pathogen by use of King's B medium broth.

Results and Discussion

The pathogen was not isolated from any seed in the ten replicas and as a result no further work was done using this technique.

Conclusion

This work on seed inoculation methods shows that a high percentage infestation occurred when seed was vacuum infiltrated. The pathogen was also recovered from seed which was artificially contaminated indicating that the pathogen can survive in association with the seed. The percentage association was not determined. The pathogen was regularly isolated from vacuum infiltrated seed and because of the rapidity and the repeatability of vacuum infiltration, this process was usually preferred to artificial contamination methods. Artificial contamination was used in some of the initial work prior to the perfection of the infiltration process.

An inoculation method, simulating the possible natural association of the pathogen with the seed would be advantageous to this study as it would enable all work with inoculated seed to be more closely related to what may occur naturally. Because two methods intending to simulate this were tried without success, vacuum infiltration was used in most seed inoculation work.

3.2.2 Isolation and Identification from seed

A number of techniques were experimented with in an attempt to develop a method which could be used to isolate and/or identify the pathogen, when it is believed to be in association with the seed. Such a technique should, primarily, be accurate for all identifications. However, other criteria including sensitivity, simplicity, rapidity and reproducibility of the method should also be considered. In this work all techniques were initially tested, using artificially infested seed, to determine the efficiency of the technique. If the method was successful it was then tested with naturally infested or commercial seedlines.

(a) Seed germination

Many seed-borne pathogens and, in particular, some bacteria, can be readily detected from seed by germination tests. Germination of seeds under conditions favouring the development of disease on the seedlings may demonstrate the presence or absence of the pathogen in a seedline. However, it would not accurately identify the number of seeds infested as diseased seedlings may not result from all infested seeds and spread between seedlings may occur soon after germination.

Bryan (1933) reported that infected cotyledons resulted when seed, contaminated with *P.syringae* pv *tomato*, was germinated. Alstatt (1944), Reid (1948), and Chambers and Merriman (1975) implied diseased seedlings resulted from the association of the pathogen with the seed. However, work by Grogan *et al* (1974) failed to demonstrate the presence of the bacterial speck organism on any plants grown from artificially contaminated seed. After the commencement of this study, Bashan *et al* (1978) published a paper which suggested the regular appearance of infected seedlings, germinated from 2 out of 3 commercial seedlots, was due to presence of *P.syringae* pv *tomato* as a surface contaminant on the tomato seed. More recently Kim (1979) reported

that seeds carrying *P. syringae* pv *tomato* produced infected plants after one month in a moist chamber.

Materials and Methods

Five hundred seeds of each of seven commercial tomato cultivars viz: 'Castlong', 'Dorchester', 'Fireball', 'UC 134' and 'VF 145-B-7879' and two 'Scoresby' seedlines were germinated at 20-25C in soil in a glasshouse, in soil in a mist chamber at 25C and on blotters in a 'Copenhagen' seed germination tank at 25C.

Five hundred seeds of each of four of these seedlines, 'Castlong', 'Dorchester', 'UC 134', and 'VF 145-B-7879' were vacuum infiltrated with the pathogen and germinated in the three environments described.

As well as these germination tests, the germination of many thousands of tomato seeds in two commercial nurseries, were observed at least once a week throughout the late winter and spring of 1979. Five cultivars; 'Beefsteak', 'Grosse Lisse', 'Moneymaker', 'Russian Red', and 'Scoresby' were the main varieties being produced at these nurseries.

Results and Discussion

No lesions typical of bacterial speck were apparent on any of the cotyledons or first leaves of seedlings germinated from the commercial or vacuum infiltrated seedlines. No symptoms were apparent on any of the seedlings in the commercial nurseries until after pricking out. The disease development on older seedlings can not be directly attributed to seedborne inoculum as other sources of inoculum may exist.

Although no infected seedlings resulted from the germination of vacuum infiltrated or commercial seedlines this does not imply the pathogen is not seedborne. The pathogen may only infect the developing plant, from the seed, under certain favourable conditions.

All the germination tests indicate is that germination of the seed is probably of little use when endeavouring to identify infested seedlines.

(b) Direct isolation method

A single infested seed usually contains large numbers of pathogenic bacteria (Taylor 1978). These pathogenic bacteria can be readily isolated by extraction from the seed and plating to a suitable medium, without prior incubation or enrichment. The National Vegetable Research Station's (NVRS) method for isolating from seed, as described by Taylor (1978), was used in this study.

Materials and Methods

One hundred seeds (25 per sample) of each of 4 artificially contaminated seedlines, 'Castlong', 'Dorchester', 'UC 134' and 'VF 145-B-7879', were ground using a pestle and mortar and the flour was suspended in 1 ml of sterile distilled water. The suspension from each seed sample was streaked to 5 agar plates of King's B medium and incubated at 25C for 36 h. All blue fluorescent colonies were tested for oxidase production and all oxidase negative colonies were tested for pathogenicity to tomatoes.

Results and Discussion

The pathogen was isolated from one sample of the 'Castlong', 'Dorchester' and 'VF 145-B-7879' seedlines, respectively.

Taylor (1978) reported that the NVRS method will detect 10^2 bacterial/ml or will detect a single *P.syringae* pv *phaseolicola* infected seed in 10,000. Although the sensitivity of this technique was not tested for *P.syringae* pv *tomato*, the pathogen was not readily isolated from the seed sample. Taylor (1978) reported that the NVRS method had no serious limitations for the

detection of plant pathogenic bacteria. However, the lack of success obtained in its use to identify *P.syringae* pv *tomato* made it apparent that a more sensitive and accurate technique was required.

(c) Enrichment assay techniques

A number of techniques using enrichment culture were tried. The usefulness of the various methods to isolate and identify the pathogen from artificially infested seed was considered prior to the use of any method in attempts to isolate from naturally infested seedlines.

Trial 1

Materials and Methods

Initially 20 whole, vacuum infiltrated or artificially contaminated, seeds of the 4 seedlines used in the NVRS method were placed directly into King's B medium broth and incubated at 25C for 24-30 h. After incubation the broth was either streaked or dilution plated to King's B medium agar plates. After 1-2 days incubation at 25C the plates were observed for fluorescence under ultraviolet light. Blue fluorescent colonies were tested for oxidase production and all oxidase negative colonies were tested for pathogenicity to tomatoes.

Results and Discussion

P.syringae pv *tomato* was regularly recovered from 'Dorchester' and 'VF 145-B-7879' seedlines and was recovered from 'Castlong' and 'UC 134' on one occasion.

This technique was successfully used to isolate and identify the pathogen from artificially infested seed but, gives no indication of how sensitive the test is. A high percentage of the seed in these samples may have been infested thus explaining the regular recovery of the pathogen.

Trial 2

The sensitivity of the above technique was tested, using both whole and ground seeds enriched in King's B medium broth.

Materials and Methods

'Fireball' seed was autoclaved (121 C for 15 mins) and individual 'VF 145-B-7879' seeds, known to be carrying the pathogen, were added to the autoclaved seed sample at the following rates: two vacuum infiltrated seeds per 500, 1000, 2000, 4000 and 8000 autoclaved seeds. Five replicas of each sample size were made. Two replicas of each sample were ground in a grinder and the flour added to 14 ml of King's B medium broth in a McCartney bottle. The remaining 3 replicas of each seed sample were added directly to King's B medium broth. Autoclaved seed was added to King's B medium broth as a control. All samples were incubated for 24 h at 25C, dilution plated to King's B medium agar and incubated for 1-2 days at 25C. All blue fluorescent colonies were tested for oxidase production and oxidase negative colonies were tested for pathogenicity on tomato seedlings.

Results and Discussion

The pathogen was only recovered from the samples containing 2 seeds/500 whole autoclaved seeds and from one sample containing 2 seeds/2000 whole autoclaved seeds. (Appendix IV, Table 30).

Although the pathogen was detected from only two sample sizes a more sensitive, more readily reproducible technique is required if the low percentage infestation, which probably occurs in a naturally infested seedline, is to be detected.

P. syringae pv *tomato* is slow growing compared to many contaminant bacteria, hence the pathogen may have been present in the sample but was unable to compete with the contaminant bacteria present. Taylor (1978) considers it essential to avoid incubation or enrichment culture of the seed sample as this favours the growth of contaminant bacteria. However, due to the lack of success attained using the NVRS method, a selective or sensitive enrichment culture technique appeared more promising for use in isolation and identification of the pathogen from the seed. Accordingly further efforts were made to develop a more sensitive and selective enrichment culture technique.

Trial 3

Materials and Methods

Vacuum infiltrated 'VF 145-B-7879' seeds, known to be carrying the pathogen, were mixed with autoclaved 'Fireball' seeds at the following rates: 10 seeds per 500, 1000, 2000 and 4000. Ten replicas of each sample size were ground in a grinder and the flour was added to sterile water at 250 g/l. Two replicas of each sample size were added directly to sterile water at the same rates. The controls were prepared by adding 2 replicas of 500 ground, autoclaved seeds to sterile water and by adding approximately 50 bacteria from a 10^3 cell/ml bacterial suspension of *P. syringae* pv *tomato* to 2,4,8 and 16 ml of sterile water.

All water suspensions were shaken overnight on an orbital shaker at 80 rpm and then filtered through Whatman No. 1 filter paper. The resultant suspension was spun for 10 min at 12,000 g. The supernatant was poured off directly onto King's B medium agar plates for some of the ground seed samples and was discarded for all other samples. The pellet was re-suspended in D4 broth, incubated 25C for 24 h and sub-cultured to 6 King's B medium agar plates. All media plates were incubated 25 C for 1-2 days and blue fluorescent colonies were tested for oxidase production, with oxidase negative colonies being tested for pathogenicity.

Results and Discussion

The pathogen was only recovered from the controls, in which approximately 50 bacteria were added to 2, 4 or 8 ml of sterile water and from the supernatant liquid from a ground seed sample containing 10 vacuum infiltrated seeds/2,000 autoclaved seeds (see Appendix IV, Table 31).

Although this technique was used to isolate the pathogen from water samples in which the pathogen was present at approximately 5.5 bacteria per ml, it was only successfully used to isolate the pathogen from one seed sample known to be carrying the pathogen. The pathogen was detected in the supernatant of a sample, after incubation, but prior to the enrichment culture. Although the selective D4 medium was used, a large number of contaminants were isolated. Any of the slow growing *P. syringae* pv *tomato* present in the culture would probably compete unfavourably with the contaminant bacteria. It had been hoped that the use of D4 broth, which shows some selectivity for pseudomonads, would have reduced the problem encountered with contaminant bacteria in previous tests.

The suspension obtained after filtration was spun to concentrate most of the bacterial cells in the pellet and the supernatant was sampled to determine if all the cells had been pelleted. The pathogen may have been isolated from the supernatant because the bacterial cells were less concentrated so each individual cell produced colonies on King's B medium agar and *P. syringae* pv *tomato* was not excluded due to competition.

If an enrichment culture technique is to be useful in the isolation and identification of the pathogen from the seed it is obvious it will need to be more selective and sensitive than the method used and reported herein.

Trial 4

Schneider (personal communication 1979) was able to isolate *P. syringae* pv *tomato* from a sample, containing 1 artificially infested seed in 10,000, using a technique similar to that outlined in this section.

Materials and Methods

Vacuum infiltrated 'VF 145-B-7879' seed, known to be carrying the pathogen, was mixed with a commercial seedline 'Fireball' (believed to be pathogen free) at the following rates: 8 seeds/500, 1,000, 2,000, 4,000 and 8,000 seeds; 4 seeds/8,000 seeds; 2 seeds/8,000 seeds and 1 seed/8,000 seeds. Two replicas of each seed sample were added to 14-20 mls of D4 broth which had been made more selective by the addition of 2.5 g/l of LiCl (Appendix I). The commercial seedline was added directly to D4 broth as a control. A bacterial suspension of *P. syringae* pv *tomato* was also added to D4 broth at approximately 400 bacteria/14 ml and 4 bacteria/14 ml, to provide further controls. All enrichment cultures were incubated on an orbital shaker at 80 rpm until the broth became cloudy, turned the consistency of a thin oil, or for up to 10 days. Each broth culture was then streaked and dilution plated to six D4 agar plates and incubated at 25C for up to 7 days. The D4 agar was made more differential by the addition of 1 ppm crystal violet. (Appendix I).

Schneider (pers. comm. 1979) reported that *P. syringae* pv *tomato* produced distinctive colonies on D4 agar + crystal violet so the differential ability of the medium was determined by plating out 3 isolates of *P. syringae* pv *tomato* (C3, C5 and C7), an isolate of *P. cichorii* (i), *P. solanacearum* and 3 contaminating isolates (I1, I2 and I3) (Appendix III) and comparing colony morphology. Unfortunately all isolates, other than *P. solanacearum* which failed to grow, produced colonies with similar morphology after 3-4 days.

All colonies from the seed sample which resembled *P.syringae* pv *tomato* in colony morphology, were sub-cultured to King's B medium agar, incubated at 25C for 1-2 days and observed under the ultraviolet light. Blue fluorescent colonies were tested for oxidase production and oxidase negative colonies were tested for pathogenicity on tomatoes.

Results and Discussion

The pathogen was readily recovered from the controls when either 400 or 4 bacteria were added to 14 ml of D4 broth (Appendix IV, Table 32).

Neither the pathogen nor any contaminant bacteria grew on the D4 agar after enrichment culture in D4 broth. Green fluorescent contaminant colonies were isolated from two of the eight samples when the D4 broth was dilution plated to King's B medium agar.

The technique was successfully used to isolate and identify the pathogen when it was added to D4 broth in low concentrations. The *P.syringae* pv *tomato* colonies did not have the distinctive colony morphology on D4 agar reported by Schneider (pers. comm. 1979) and the technique was not successfully used to detect 8 artificially infested seeds in 500 let alone the 1 artificially infested seed in 10,000 reported. Why this technique failed in this study is not known but, with the sensitivity of the technique experienced herein, it is obvious a more sensitive technique is required.

Trial 5

Materials and Methods

Cooke's microtitre plates were surface sterilised by immersion in 70% alcohol for 10-20 min., and dried in a laminar flow cabinet. A few drops of King's B medium broth and one vacuum infiltrated 'VF 145-B-7879', 'UC 134' or 'Dorchester' seed was added to each of

the 96 wells in the microtitre plates (Plate 9). The plates were incubated in stacks, at 25C for 24 h with a sterile empty plate on the top to maintain aseptic conditions. After incubation 1 loop full of each broth culture was streaked to a King's B medium agar plate divided into 12 sections (Plate 10) and incubated at 25 C for 1-2 days. All blue fluorescent colonies were tested for oxidase production with all oxidase negative colonies being tested for pathogenicity to tomatoes. Individual infested seeds were removed from the broth culture, dried and stored.

This technique was used to screen seed extracted from infected fruit and commercial seedlines, suspected of carrying the pathogen, as well as artificially infested seedlines.

Diseased fruit of 'Beefsteak', 'Castlong' and 'Scoresby' varieties were collected from crops in the Manawatu and Hawkes Bay regions. The seed was hand extracted from individual diseased fruits with over 50 speck lesions, by cutting them in half and squeezing the pulp into a 12 mesh 1.4 mm aperture strainer. The pulp was washed through the strainer with sterile water and the seed was dried on blotting paper at room temperature. Dried seeds were placed in wells in Cooke's microtitre plates and tested for the presence of the pathogen in the same manner as described previously.

Seedling tomato plants in 2 commercial nurseries in Palmerston North were observed weekly from emergence through until sale throughout the late winter and spring of 1979. The disease appeared in both nurseries in the first week in October 1979. The incidence of the disease was very high in both nurseries. In one nursery nearly all 'Moneymaker' seedlings, from a seedline acid extracted by the nursery from a locally grown crop, were infected. The seedlings of 'Potentate', and 'Scoresby' (Webling and Stewart) also showed low percentage infections. In the other nursery the disease was predominant in 'Beefsteak' seedlings (Ocean View Seeds Ltd; fungicide treated) with the occasional infected 'Grosse Lisse' or 'Russian Red' seedling also being encountered.

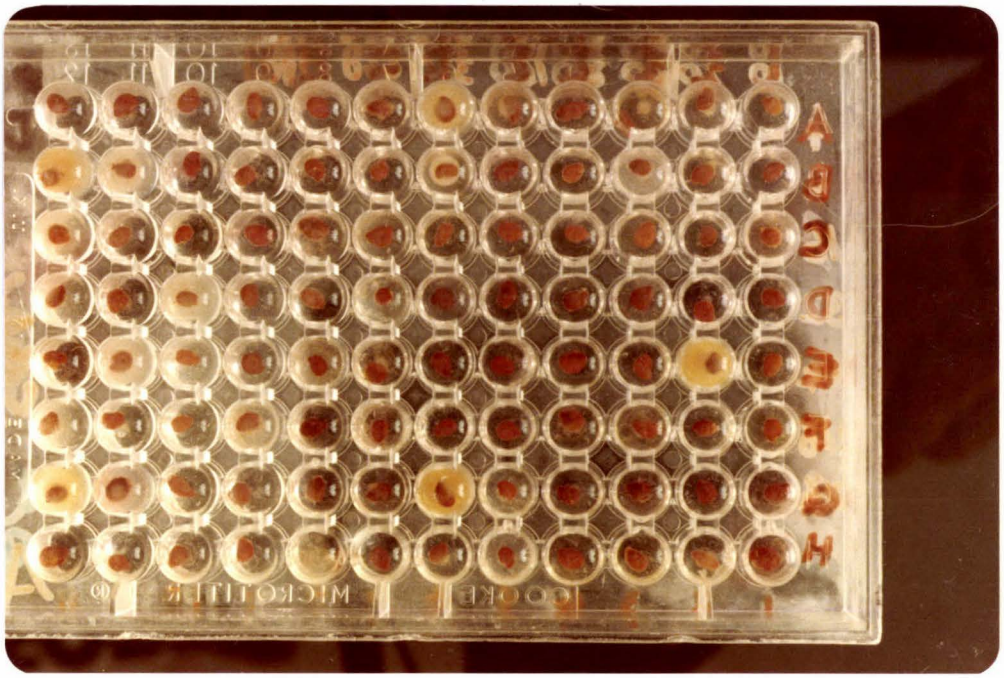


Plate 9: Cooke's microtitre plate with a tomato seed and King's B medium broth in each well.

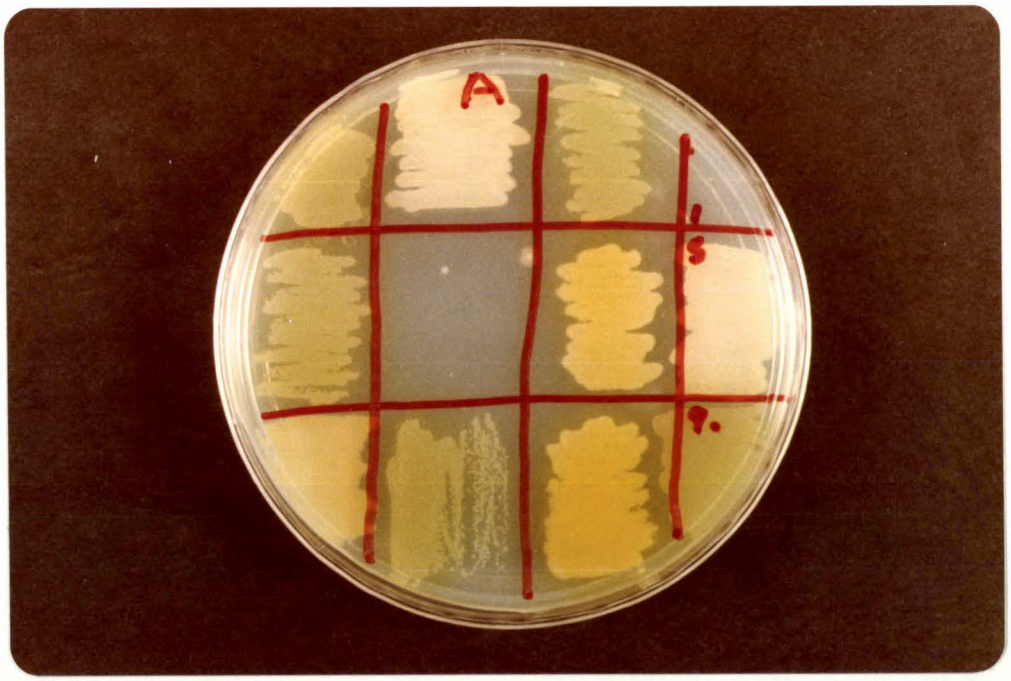


Plate 10: King's B medium agar streaked with 12 enrichment cultures from a Cooke's microtitre plate.

One 'Moneymaker' seed was added to each well of 4 Cooke's microtitre plates. The 'Potentate', 'Scoresby' and 'Beefsteak' seedlines were tested by adding 1 seed to each well of 3 Cooke's microtitre plates. The 'Moneymaker' and 'Beefsteak' seedlines were also tested by adding 5 seeds to each well of the microtitre plates. These two seedlines were tested more extensively than the other seedlines because the seedlings showed a high percentage infection in the nursery, which may be correlated with the pathogen being seedborne. All wells were tested in the manner described earlier. Five seedlines, 'Castlong', 'Dorchester','UC 134', 'VF 145-B-7879' and 'Fireball' were also tested.

Results and Discussion

The pathogen was regularly isolated from vacuum infiltrated seedlines, with a high percentage of the seeds being infested in some cases (Tables 5 and 6).

Table 5: Isolation and identification of *P.syringae* pv *tomato* from vacuum infiltrated seedlines using the Cooke's microtitre plate system

Cultivars	No. of seeds tested	Blue fluorescent isolates	Oxidase reaction	Pathogenicity	% of seeds infested
'Dorchester'	1344	248	246-	246+	18.3
'UC 134'	192	43	43-	43+	22.4
'VF 145-B-7879'	1152	699	562-	562+	48.8

The percentage of seed infested varied considerably with each vacuum infiltration. This is well demonstrated by observing the percentage infestation in the 'Dorchester' seed samples vacuum infiltrated on different occasions (Table 6).

Table 6: Variation in percentage infestation of vacuum infiltrated 'Dorchester' seed

	No. of seeds tested	Blue fluorescent isolates	Oxidase reaction	Pathogenicity	% of seeds infested
	288	181	181-	181+	62
	384	60	58-	58+	15
	672	7	7-	7+	1.04
Total	1344	248	246-	246+	18.3*

* Average % of seeds infested

The technique was used to isolate from a high percentage of vacuum infiltrated seeds of three cultivars. The technique showed a surprisingly large variation in the number of infested seeds resulting from vacuum infiltration at different times. This difference is presumably due to some variation in the vacuum infiltration process.

Because the Cooke's microtitre plate system was successfully used to isolate and identify the pathogen from artificially infested seeds, attempts were made to isolate from seeds, believed to be, naturally infested.

The pathogen was regularly isolated from 'Beefsteak' seed, hand extracted from diseased fruit collected from a diseased crop in the Manawatu, but was never isolated from any seeds hand extracted from 'Castlong' or 'Scoresby' fruit (Tables 7 & 8). The percentage of infested 'Beefsteak' seeds varied slightly from one microtitre plate to another, with a large increase in the percentage of infested seeds appearing from the second sample of diseased fruit collected 16 days after the first.

Table 7: Isolation and identification of *P.syringae* pv *tomato* from naturally infested seedlines

Cultivar	No. of seeds tested	Blue fluorescent isolates	Oxidase reaction	Pathogenicity	% of seeds infested
'Beefsteak'	1056	266	169-	164+	15.5
'Castlong'	96	14	0	0	0
'Scoresby'	288	19	0	0	0

Table 8: Variation in the detection of infested 'Beefsteak' seeds collected at different times from the same diseased plot

Collection Date	No. of seeds tested	Blue fluorescent	Oxidase reaction	Pathogenicity	% of seeds infested
5.3.79	96	10	6-	5+	5.2
	96	8	4-	3+	3.1
	96	12	5-	4+	4.2
	96	8	3-	3+	3.1
	96	14	4-	4+	4.2
	96	15	10-	10+	10.4
	96	11	8-	6+	6.2
Total	672	78	40	35	6*
21.3.79	96	74	73-	73+	76
	96	39	16-	16+	16.6
	96	48	17-	17+	17.7
	96	27	23-	23+	24
Total	384	188	129	129	33.6*

* Average % of seeds infested

An independant t-test was performed on the figures obtained in Table 8 to determine if there is a significant difference in the percentage of infested seeds collected at each time interval. A value of 3.6 was obtained for t_i with 4 degrees of freedom. This difference is significant at the 5% level but not at the 1% level. The fruit collected at each time were at similar stages of maturity and had similar percentage infections so any difference is probably attributable to some unknown factor acting in the field. The significant difference may be due to the early exposure of fruit collected on the 21.3.79 to the pathogen and favourable environmental conditions for disease development. The readings from the Department of Scientific and Industrial Research Pamerston North Meterological Service were obtained for the period from 18.2.79 to 21.3.79 and are summarised in Table 9.

Table 9: DSIR's meterological service recordings for the 16 days prior to each collection date

Date for 16 day intervals	Average max temp. C	Average min temp. C	Average rainfall	No. of rainfall days	Average max temp on rainy days
18.2/5.3.79	19.3	11.7	2.0	5	19.3
6.3/21.3.79	22.3	14.3	5.6	8	21.7

Basu (1966) reported that disease development is favoured by 24-27C and 90-100% RH. From the results of seed infestation tests (Table 8) and the meterological data (Table 9) it appears environmental conditions between 6.3.79 and 21.3.79 were more conducive to the disease than between 18.2.79 and 5.3.79. The longer exposure to disease and the more favourable environmental conditions for disease development may account for the significant increase in the number of infested seeds at the second collection interval.

When hand extracting the seed in this work care was taken to avoid contact between the lesioned fruit surface and the seed extract. Grogan *et al* (1974) and Bashan *et al* (1978) both reported the pathogen was not systemic and fruit lesions are superficial (Dye 1975). In this hand extraction process there was no contact between the seed and the fruit surface so it is not known how the seeds became infested.

This work has established that the pathogen can naturally infest the seed and could therefore be present in a commercial seedline. Of the 9 commercial seedlines tested the pathogen was only isolated from two, viz, 'Beefsteak' and 'Moneymaker' seedlines (Table 10). The pathogen was recovered from a high percentage of the 'Beefsteak' seeds and from a low, but notable percentage, of the 'Moneymaker' seeds.

Table 10: Isolation and identification of *P. syringae* pv *tomato* from commercial seedlines using the Cooke's microtitre plate system

Variety	No. of seeds tested	No. of seeds/ well	No. of blue fluorescent isolates	Oxi- dase reaction	Patho- genic- ity	min% of seeds infested
'Beefsteak'	192	1	0			0
'Moneymaker'	384	1	0			0
'Potentate'	288	1	0			0
'Scoresby'	288	1	0			0
'Beefsteak'	480	5	36	36-	36+	7.5
'Moneymaker'	1440	5	2	2-	2+	0.14

If *P.syringae* pv *tomato* can infect developing seedlings as a result of its association with the seed, then this could explain the high percentage of infected seedlings observed in the two nurseries using these seedlines. Although the percentage of 'Moneymaker' seeds from which the pathogen was isolated is low, it only requires one infested seedling to result from the pathogen being seedborne and the disease may spread rapidly reaching epidemic proportion under favourable environmental conditions.

This detection technique was successfully used to both isolate and identify the pathogen from the seed when other enrichment culture techniques failed, probably because *P.syringae* pv *tomato* was present in the wells in higher concentrations than contaminant bacteria, thus enabling it to compete favourably.

The Cooke's microtitre plate system fulfils some of the criteria required for a technique to be useful in isolation and identification of *P.syringae* pv *tomato*. It is an accurate, sensitive, repeatable and simple technique. However, it fails to fill one important criterion i.e. rapidity. Unfortunately the technique is rather time consuming thus it is impractical to use it to screen large seedlines but, despite its faults it was the most promising technique tried and was instrumental in establishing beyond doubt that *P.syringae* pv *tomato* can be seedborne.

(d) Agar plug technique

Speirs (pers comm 1980) used a leaf disc technique to test the susceptibility of poplar to *Marsonina* spp. and to determine the environmental conditions favouring disease development. A similar technique was tested in this study.

Materials and Methods

Holes were made in thick 2% water agar plates (Appendix I) with a size 7 corkborer and the same size corkborer was used to make leaf discs from disease free tomato leaves. One tomato leaf disc was put in each hole in the water agar plate, 3-5 moist tomato seeds were added (Plate 11) and the plates were incubated in the light, at 25C for 10 days.

Seed germination can be prevented or retarded by soaking the seed in 2,4-D (Wood 1966) for 20 mins. The seed was soaked in 0.025%, 0.05% and 0.1% 2,4-D and placed on the leaf discs to determine the most useful concentration for this work.

Initially the technique was tested by adding 1 drop of a suspension of *P. syringae* pv *tomato* to the upper and lower leaf surfaces of the discs. The plates were incubated and observed daily for symptom production. After 7 days incubation the leaf discs were removed from the wells and isolations were made either by macerating each leaf disc in sterile water and streaking to King's B medium or by enriching the leaf disc in D4 broth and subculturing to King's B medium agar. In this work all King's B medium agar was supplemented with 200 units of penicillin per 10 ml of agar (Bashan *et al* 1978) to increase its selectivity.

Moist vacuum infiltrated 'VF 145-B-7879' or 'Dorchester' seeds were deposited 3/leaf disc on both young and old leaf discs and on both lower and upper leaf surfaces. A control leaf disc, to which no seeds were added, was placed in each plate. It was not necessary to treat the seeds to inhibit germination unless the plates were incubated for more than 7 days.

As well as testing bacterial suspensions and vacuum infiltrated seedlines the technique was used to test 'Beefsteak', 'Moneymaker' and 'Scoresby' commercial seedlines thought to be carrying the pathogen and the technique was compared with the Cooke's microtitre plate system.

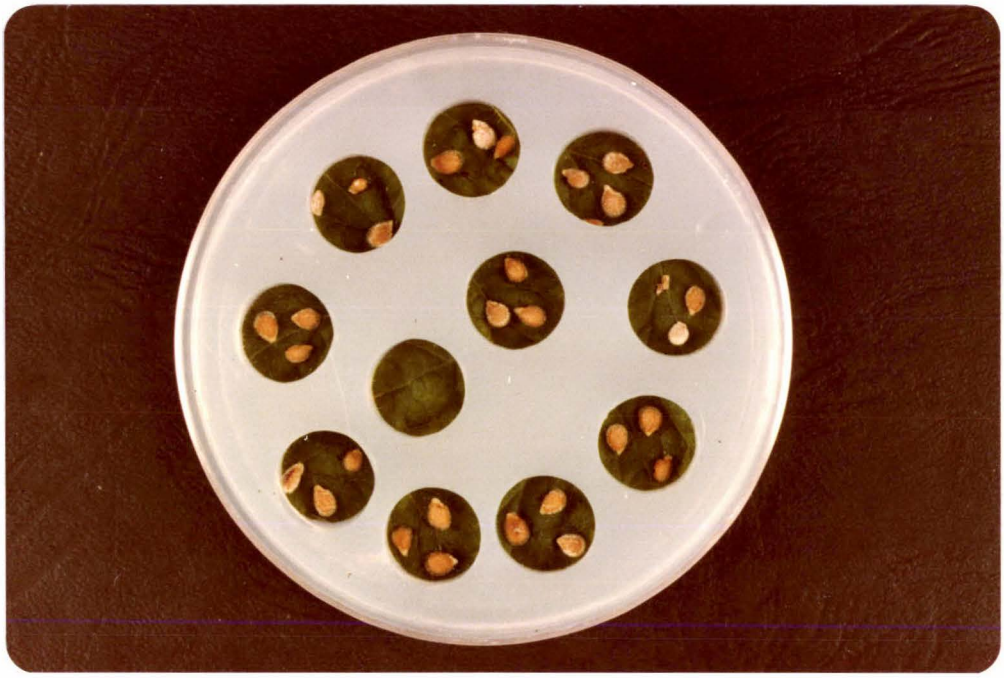


Plate 11: Tomato seeds on tomato leaf discs embedded in 2% water agar.

Results and Discussion

No speck lesions were apparent on any of the leaf discs after 7 days incubation, at which time the leaves began to dry and curl around the edges making the identification of any speck lesions appearing after this time difficult. It was unfortunate that the identification of the pathogen could not be made by observing symptom production. However, *P. syringae* pv *tomato* was isolated from all inoculated leaf discs by maceration and enrichment culture of the leaf discs. Because the leaf discs were not surface sterilised no conclusion could be drawn as to whether the bacteria infected the leaf or simply survived on the surface.

Seed germination can be prevented or retarded by soaking the seed in 2,4-D (Appendix IV, Table 33). All concentrations of 2,4-D considerably reduced the percentage germination of the seed while usually having no obvious effect on the tomato leaf discs. Some of the leaf discs turned brown and necrotic when the seed was treated with 0.1% 2,4-D and no seeds germinated. Only 3% of the seeds treated with 0.025% and 0.05% 2,4-D germinated and neither concentration had any obvious effect on the leaf discs. As both 0.025% and 0.05% 2,4-D had a similar effect on seed germination and the leaf discs either concentration could be used when it is necessary to prevent or retard seed germination.

P. syringae pv *tomato* was regularly isolated from leaf discs upon which vacuum infiltrated 'Dorchester' or 'VF 145-B-7879' seed had been sown (Table 11). The percentage of leaf discs infested and the minimum percentage of seed infestation were calculated in each case. The pathogen was also isolated and identified from 3 commercial seedlines (Table 12).

Table 11: Isolation and identification of *P.syringae* pv *tomato* from leaf discs inoculated with vacuum infiltrated seed

Treatment	Replicas	No. of seeds tested	No. of blue fluorescent isolates	Oxidase reaction	Pathogenicity	% of discs infested	min% seed infested
VF 145-B-7879 upper	15	45	3	3-	3+	20	6.6
VF 145-B-7879 lower	15	45	2	2-	2+	13.3	4.4
Dorchester old leaf	26	78	9	9-	6+	23.0	7.6
Dorchester young leaf	47	141	20	20-	20+	42.5	14.2
Controls (no seed)	7	0	0			0	

Table 12: Isolation and identification of *P.syringae* pv *tomato* from leaf discs inoculated with commercial seedlines

Seed-line	Replicas	No. of seeds tested	No. of blue fluorescent isolates	Oxidase reaction	Pathogenicity	% of discs infested	min % of seeds infested
'Beefsteak'	100	500	14	2-	2+	2	0.4
'Moneymaker'	100	500	18	7-	7+	7	1.4
'Scoresby'	86	430	14	1-	1+	1.2	0.23
Controls	20	0	0			0	

This technique would be more useful it if was possible to identify the pathogen by symptoms produced on the leaf disc. The agar plug method does not seem to be as sensitive as the Cooke's microtitre plate system, although *P.syringae* pv *tomato* was detected in a 10 fold higher percentage of 'Moneymaker' seed and was also detected in a

low percentage of 'Scoresby' seeds using the agar plug technique.

Because of the time consuming and laborious nature of this technique its uses were limited and the Cooke's microtitre plate system was generally preferred for use in isolation and identification of *P. syringae* pv *tomato* from the seed.

(e) Serology

Most serological techniques require a minimum concentration of antigen to be readily available for reaction with the antibodies. If *P. syringae* is *tomato* associated with the seed it probably fulfils neither of these criteria. Most serological tests are made using cultures of the antigen. However, it was hoped that the use of serological tests in this study would eliminate the need to isolate and culture the bacteria when attempting to identify infested seeds. If the serological test required the culture of the pathogen it would be more sensible to use the differential and accurate pathogenicity tests in an identification.

Three serological tests were considered for use in this study, viz; gel double diffusion tests, direct or indirect fluorescent antibody tests and the ELISA (Enzyme-linked immunosorbent assay) techniques. The direct or indirect fluorescent antibody tests require that all reactions are species specific. However, the main problem may be getting the antibodies to contact a few antigens which may be 'trapped' within the seed. This technique was not considered of any use for this work. Although it is extremely sensitive, the ELISA technique would require antigens to be concentrated so would probably not detect the low concentration of antigens in the seed. Gel double diffusion tests are the only tests which may be useful in this situation because a positive result is determined by the appearance of a precipitate resulting from the contact of antibodies and antigens when they diffuse through the agar from their sources.

Materials and Methods

Single wells were made in a 0.4% phosphate buffered saline serology agar plate (PBSSA) (Appendix I) and filled with a 1:4 dilution of antisera to *P.syringae* pv *tomato* with 0.85% sterile saline. Vacuum infiltrated 'VF 145-B-7879' and 'Dorchester' seedlines, known to be carrying the pathogen, were either repeatedly frozen and thawed or heated to 100C for 20 mins to destroy all heat labile non-specific antigens. One hundred seeds of each cultivar for each heat treatment were submerged in PBSSA in circles surrounding the central antisera wells, incubated in a humid container at 25C for 5 days and observed using back illumination. Any seeds reacting with the antisera, as indicated by the appearance of a fine precipitate zone in the agar, were tested by use of the Cooke's microtitre plate system for the presence of *P.syringae* pv *tomato*.

Results and Discussion

No obvious precipitate lines appeared around any of seeds. Some of the seeds were tested for the presence of *P.syringae* pv *tomato* because a very faint precipitate formed underneath the seed in the agar, but none of these seeds were found to be carrying the pathogen.

Because most antigens were not released from the seed the low concentrations of antigen diffusing into the agar were probably insufficient to react with the antibodies so that a visible precipitate resulted. From previous work with serology it is known that both antigen and antibody can diffuse through the agar and a visible precipitate results. A visible precipitate failed to appear around the seeds indicating either the pathogen is not present in the seed or that low concentrations of antigen cannot be detected.

Serology could be used to identify the pathogen after isolations had been made. However, this requires the culture of the pathogen, an action, it was hoped, serological tests could avoid. If there

was a high population of the pathogen, in association with the seed, which could be released into the agar it might be possible to detect the antigen associated with the seed by gel double diffusion tests.

(f) Ultraviolet light technique

Both Wharton (1967) and Parker and Dean (1968) reported that the seeds of white and light seed-coated bean varieties infected with *P. syringae* pv *phaseolicola* fluoresced when viewed under ultraviolet light. Fluorescence was not observed in dark seed-coated varieties and in the seeds of varieties which had coloured seed dressing. Parker and Dean (1968) reported that the type of fluorescence varied. Seeds fluorescing bluish-white or chalky-white could not be distinguished from non-fluorescent seeds in visible light. However, seeds fluorescing orange or bright greenish yellow generally had seed-coat injury which was apparent in visible light.

If infested tomato seeds fluoresced under ultraviolet light this could possibly enable seedlines to be screened rapidly for *P. syringae* pv *tomato* infestations.

Materials and Methods

An ultraviolet emission source, which floods a wide area with light of 3500 ^{o}A was installed in a darkened room. Seeds from the following seedlines: 'Scoresby', and 'Beefsteak' hand extracted from diseased fruit, 'Potentate' and 'Moneymaker' from commercial seedlines which may have been harboring the pathogen and vacuum infiltrated 'VF 145-B-7879' were observed under the ultraviolet light. Fluorescent and non-fluorescent seeds were selected from each seedline and tested for the presence of *P. syringae* pv *tomato* by use of the Cooke's microtitre plate system.

Results and Discussion

Some of the seeds fluoresced bluish-white while others fluoresced purple or orange-brown. Care had to be taken to ensure that the fluorescent part of the seed was not grit or dirt which readily adhered to the seed-coats of hairy varieties. The seeds fluorescing white-blue were usually whole seeds while most of those fluorescing purple or orange-brown were broken or injured seeds. The pathogen was isolated from the vacuum infiltrated fluorescent and non-fluorescent seeds (Table 13).

Table 13: Isolation and identification of *P.syringae* pv *tomato* from both fluorescent and non-fluorescent seeds .

Seedline	No. of seeds tested	Blue fluorescent isolates	Oxidase reaction	Pathogenicity	% of seeds infested
'VF 145-B-7879	20	2	2-	2+	10
Fluorescent 'VF 145-B-7879'	24	9	9-	9+	37.5
'Beefsteak'	12	0			
Fluorescent 'Beefsteak'	12	0			
'Moneymaker'	30	0			
Fluorescent 'Moneymaker'	30	0			
Potentate	30	0			
Fluorescent 'Potentate'	30	0			
Fluorescent 'Scoresby'	24	1	+		

The bluish-white fluorescence of seeds detected under the ultra-violet light was often pale and indistinct making it difficult to determine if seeds were actually fluorescing. The pathogen was recovered from a high percentage of the fluorescent vacuum infiltrated

seeds, some of which were broken or injured fluoresced an orange-brown colour as well as a bluish-white. From the very limited number of vacuum infiltrated seeds tested and the high percentage of bluish-white fluorescent seeds which were infested in this sample, it appears that this may be a useful technique for reducing the number of seeds screened for the pathogen. For a technique, such as this, to be useful it must be able to detect some of the infested seeds in seedlines which are believed to be naturally infested. For this reason most of the fluorescing seeds tested for the pathogen were from hand-extracted or commercial seedlines. Unfortunately no seeds from these seedlines were naturally infested with the pathogen.

Fluorescence could only be detected in varieties with light coloured seed-coats. Due to the small size of the seed and the indistinctness of the small, usually peripheral fluorescence, detection of fluorescing seeds was difficult. Although the technique appeared promising for vacuum infiltrated seed, it was of no use in screening naturally infested seedlines for *P.syringae* pv *tomato*.

Conclusion

A number of isolation and identification techniques were evaluated in an attempt to develop a technique which is sensitive, simple, rapid and reproducible for the isolation and identification of *P.syringae* pv *tomato* from seed. Most of the techniques lacked sensitivity and only two techniques were successfully used to repeatedly isolate *P.syringae* pv *tomato* from the seed. Both these techniques were simple, but unfortunately both were very time consuming. The Cooke's microtitre plate system was more sensitive than the agar plug technique. Both methods were used to isolate and identify *P.syringae* pv *tomato* from vacuum infiltrated and commercial, naturally infested seedlines. The minimum percentage infestation of 2 commercial seedlines was determined using the Cooke's microtitre plate system and the minimum percentage infestation of 3 commercial seedlines was determined by the agar plug technique.

Initially, when experimental work commenced, attempts to isolate from leaf lesions by tissue plating were largely unsuccessful because of the other bacteria competing with *P.syringae* pv *tomato*. No attempts were made to isolate and identify *P.syringae* pv *tomato* from the seed, by plating whole seeds to King's B medium agar because it was considered that an approach involving the emersion of seeds in selective media would result in the detection of more of the infested seeds. After the completion of the experimental work for this thesis, an abstract (Kim 1979) was published reporting that *P.syringae* pv *tomato* could be isolated from infested seeds by incubating the seeds on King's B medium agar for 2-4 days at 26C.

The seed plating technique is probably a little less sensitive than the Cooke's microtitre plate system, because the pathogen may not be detected if it infests the seed parts not contacting the King's B medium, whereas the whole seed surface contacts the King's B medium broth in the wells in Cooke's microtitre plates. Although the seed plating technique (Kim 1979) may be less sensitive than the Cooke's microtitre plate system, it is a less time consuming technique and, may be more useful for the isolation and identification of *P.syringae* pv *tomato* from individual seeds than any of the methods used in this study.

3.3 Seed-Seed Transfer

The term seed-seed transfer is regularly used to describe the spread of the pathogen from seed to seed, either in the seed cleaning process or during storage. In this study the term will also be extended to include the time between seed harvest and seed sowing, or the ability of the pathogen to survive seed storage. Seed treatments are applied some time between harvest and sowing and so should, ideally, be included in this section. However, it is essential to establish that the pathogen can be seed-borne before discussing seed treatments, so although they will not be discussed in any detail in this section it is important to remember that seed treatments may have a deleterious effect on the survival of *P.syringae* pv *tomato* in association with the seed.

Although the term seed-seed transfer is not used in its usual context, it is possible that the pathogen could infest one seed from another during storage. However, because only a low percentage of seeds have been found to be naturally infested with *P.syringae* pv *tomato* seed to seed spread is probably of little importance otherwise a higher percentage infestation could be expected.

Survival

In work on survival it was essential to know that the seed used in the experiments was infested with *P.syringae* pv *tomato* and also when infestation occurred. Although some work was done on survival in naturally infested, hand extracted seed and in commercial seedlines, artificially inoculated seed was used in most work because the precise date of infestation of these seeds was known.

Materials and Methods

Initially it was essential to establish that *P.syringae* pv *tomato* could survive in association with tomato seed for even very short periods of time.

Three grams of artificially contaminated seeds of four varieties viz. 'Castlong', 'Dorchester', 'VF 145-B-7879' and 'UC 134' were sampled (3 replicas of 20 seeds for each variety) at one day intervals for 3 days, then at 3 day intervals for 24 days. 'Dorchester' and 'VF 145-B-7879' were then sampled at weekly intervals up until the 68th day. Three replicas of vacuum infiltrated 'Dorchester' and 'VF 145-B-7879' seed were also sampled 4,6,11,15 and 20 months after inoculation. Testing of the other seedlines was discontinued after 24 days because the seed supply was limited and the pathogen was rarely isolated from the discarded seedlines.

All inoculated seed was dried on blotting paper at room temperature or in a 16C refrigerated incubator after inoculation. When dried the seed was stored in petri plates on the laboratory bench until tested. Hand and acid extracted dried seed samples were stored in sealed polythene bags at room temperature. A commercial 'Moneymaker' seedline was stored at 4C in paper bags while not being used, but between August 1979 and February 1980 it was stored at room temperature. A commercial 'Beefsteak' seedline was stored in sealed foil sachets after harvesting and then in paper bags at room temperature between August 1979 and February 1980 when the seedline was being used.

All seed samples were assayed by introducing 20 seeds into King's B medium broth, incubating for 48 h at 25C and then streaking each enrichment culture to 3 King's B medium agar plates. The plates were incubated at 25C for 1-2 days. All blue fluorescent colonies were tested for oxidase production, with oxidase negative colonies being pathogenicity tested to tomatoes.

The hand and acid extracted seedlines were extracted from diseased fruits and tested 12 months after extraction. Commercial 'Moneymaker' and 'Beefsteak' seedlines were tested by use of the Cooke's microtitre plate system and the agar plug technique. Although the extraction date was not known it was estimated that the seed had been stored for at least 11 months, this being the interval since the previous New Zealand tomato harvest.

Results and Discussion

P. syringae pv *tomato* survived in a viable state on 'Castlong', 'Dorchester' and 'VF 145-B-7879' and was regularly isolated from the latter two seedlines throughout the duration of the experiment (Tables 14 & 15). The pathogen survived in association with the 'Dorchester' seed for 11 months at which time the artificially infested seedline was depleted. The pathogen survived in association with the 'VF 145-B-7879' seedline for 15 months, at which time the seed supply was depleted.

Table 14: Isolation of *P. syringae* pv *tomato* from seeds of four artificially infested tomato seedlines

Variety	No. of days after infestation of the seed										
	0	1	2	3	6	9	12	15	18	21	24
'Castlong'	-	-	-	+	-	-	-	-	+	-	-
'Dorchester'	+	+	+	+	+	+	+	-	-	+	+
'UC 134'	-	-	-	-	-	-	-	-	-	-	-
'VF 145-B-7879'	+	+	-	+	+	+	+	+	-	+	+

Table 15: Isolation of *P. syringae* pv *tomato* from artificially infested 'Dorchester' and 'VF 145-B-7879' seedlines

Variety	Time after infestation											
	Days							Months				
	28	35	42	49	54	61	68	4	6	11	15	20
'Dorchester'	+	-	-	+	+	+	+	-	-	+		
'VF 145-B-7879'	+	-	-	-	+	+	+	+	+	+	+	-

The pathogen was not isolated from either the hand or acid extracted 'Beefsteak' or 'Scoresby' seedlines after 12 months storage although it was isolated from the hand extracted 'Beefsteak' seedline soon after extraction. The pathogen was isolated from a 'Moneymaker' seedline acid extracted by a local nursery in March 1979, eleven months after harvest. Similar results were obtained using a commercial 'Beefsteak' seedline.

P.syringae pv *tomato* can remain viable, between harvest and sowing, in association with both naturally and artificially infested seedlines, under the storage conditions used in this work. The effect of storage on the percentage of seeds infested was not investigated, because the Cooke's microtitre plate system had not been developed when survival tests commenced. However, it is anticipated that a reduction in the percentage of infested seeds would occur when storage periods are prolonged.

Probably soon after extraction or infestation there would be a large drop in the number of viable bacteria in association with the seed, as all the bacterial speck organisms which are unable to survive desiccation would lose their viability. After this initial, large drop in the number of viable bacteria associated with the seed (a drop which may also correspond with a reduction in the number of infested seeds) it is expected that a gradual decline in the number of seeds infested with the bacteria would occur. It may be possible to 'control' the pathogen associated with the seed by prolonging seed storage, providing the viability of the bacteria is lost before seed viability falls below an acceptable level. Unfortunately, it was not possible to investigate how long the pathogen can survive in association with the seed because of the limited duration of this study.

In this section no work was conducted to establish how the pathogen is associated with the seed or to determine if any seed-seed spread occurred. It is likely that the pathogen can survive as a seed contaminant and probably can also infect the seeds. Bryan (1933) reported that *P.syringae* pv *tomato* is very sensitive to drying, especially when favourable conditions for growth are followed by dry spells. In the hairy seeded varieties bacteria may be protected from desiccation by the seed hairs and may survive in association with or infect the seed through injured hairs, in the same way as its survival has been associated with the leaf trichomes (Schneider and Grogan 1977b). In the smooth seed coated varieties survival as a contaminant could probably be compared to survival on a glass slide.

Bryan (1933) reported that almost all cultures, dried for 48h on glass slides failed to survive, with only one slow growing strain being able to survive 9 days drying. The pathogen may survive for prolonged periods in association with cracked or damaged seeds which provide a protected environment where the bacteria can survive.

In this study the pathogen was demonstrated to survive for long periods of time in association with artificially infested, smooth seed-coated varieties, either as a contaminant, or probably in damaged artificially infected seeds. The commercial 'Moneymaker' seed has rough, hairy seed coats but the fungicide treated commercial 'Beefsteak' seed had a smooth seed coat. The rough, hairy seed coat of the 'Moneymaker' seedline may enable the pathogen to survive as a contaminant and the fungicide on the 'Beefsteak' seedline may have protected any contaminant bacteria from desiccation.

Bashan *et al* (1978) reported that *P.syringae* pv *tomato* is carried as a seed contaminant because, after "surface sterilisation" of "contaminated" seeds, they were unable to isolate the bacterium. The author of this thesis does not agree with their conclusion as their "surface sterilisation" process involved exposing "contaminated" seeds to 70% ethanol followed by 1% sodium hypochlorite, each for 30 seconds "under vacuum". The vacuum was released abruptly to favour sterilisation of the seed "surface". This process could infiltrate some of the ethanol and sodium hypochlorite into the seed and thus may inactivate any bacteria infecting the seed.

The current study only established that the pathogen can remain viable between harvest and sowing in association with both artificially and naturally infested seedlines. No conclusions can be drawn regarding how the pathogen is associated with the seed or if seed-seed spread occurs.

3.4 Seed-Plant Transfer

Having established that *P.syringae* pv *tomato* can infest the seed and survive between harvest and sowing in association with the seed, it is essential to demonstrate that seed-plant transfer can occur, thus, providing evidence that the pathogen can be seedborne. To be seedborne a pathogen must infect the tomato plant at some stage of its development as a result of its association with the seed. Usually a seedborne pathogen infects the seedling soon after germination and spreads from this plant to others. Therefore it is usually possible to identify the seedborne nature of a pathogen, by performing germination tests in an environment where the developing seedling is isolated from all other possible sources of inoculum.

A number of researchers have reported that *P.syringae* pv *tomato* can infect seedlings as a result of its association with the seed. Bryan (1933) reported that infected seedlings resulted from the germination of inoculated seed in pathogen free soil. Reid (1948) associated the high percentage of infected seedlings in seedling trays with the use of imported seed. Bashan *et al* (1978) found that speck symptoms appeared on the seedlings of 2 out of 3 commercial seedlines after germination in sterile soil under mist. Kim (1979) reported that seeds carrying *P.syringae* pv *tomato* produced plants with leaf symptoms, after one month at 17-26C in a moist chamber. None of these researchers reported germinating the seedlings in isolation from all other possible sources of inoculum. This is particularly significant if the organism is ubiquitous as Schneider and Grogan (1977a) suggest.

The ability of these researchers to obtain infected seedlings as a result of the germination of infested seed is contrary to the results obtained by Grogan *et al* (1974), Chambers and Merriman (1975) and germination tests performed earlier in this study. The inability of some researchers to demonstrate seedborne spread could result from the failure to germinate infested seeds in environmental conditions which are optimum for disease development, viz, 85-100%

relative humidity and temperatures of 24-27C. The seeds germinated in Grogan *et al's* (1974) work and in this study were grown under environmental conditions believed to favour speck development (Basu 1966, Grogan *et al* 1974, Bashan *et al* 1978). Accordingly if the disease is seedborne the seedlings should have become infected. The influence of the environment on disease could be more complicated or the environmental conditions required for seedling infection could be more critical than was previously considered.

This work aimed to demonstrate that seed-plant transfer can occur; identify the environmental conditions favouring the seed-plant transfer; and identify how the pathogen becomes associated with the seedling, taking into consideration all other possible sources of inoculum.

In order to demonstrate seed-plant transfer attempts were made to isolate the pathogen from seedlings germinated(i) in Copenhagen tanks; (ii) on blotters in sealed plastic containers; (iii) in a mist chamber; and (iv) in commercial nurseries.

(i) Germination in Copenhagen tanks

Materials and Methods

Vacuum infiltrated 'VT 145-E-7879' seeds were sown, 50 per blotter, and germinated at 20C, 25C and 30C in a 14-10h light-dark regime in Copenhagen tanks. The seedlings were sampled at the cotyledon and the first leaf stage by removing any lesioned foliage with speck-like symptoms, macerating this in sterile water and streaking to King's B medium agar and D4 agar. All King's B medium agar used in this section was made more selective by the addition of penicillin and all D4 agar used was made more differential by the addition of crystal violet. All colonies on D4 agar, with a similar colony morphology to *P.syringae* pv *tomato*, were streaked to King's B medium. All blue fluorescent colonies on King's B medium were tested for oxidase production with oxidase negative colonies being tested for pathogenicity

to tomatoes.

Results and Discussion

A high percentage of the seedlings germinated at each temperature. At 30C constant, most of the seedlings became waterlogged due to the condensation. Many of the seedlings died and because of the large number of necrotic areas on the other seedlings it was impossible to determine if a pathogen was responsible for any of the lesioning so the seedlings were discarded.

A few of the seedlings germinated at 20C and 25C appeared to have lesions, which had some slight resemblance to those produced in bacterial speck infections, on the cotyledons and first leaves 2-3 weeks after sowing. Some blue fluorescent colonies were isolated when these lesions were sampled, but they were all oxidase positive.

P. syringae pv *tomato* did not infect the developing seedlings, causing lesion development, as a result of its association with the seed, when the seed was germinated in a Copenhagen tank, possibly because the environmental conditions in the Copenhagen tank were not favourable for disease development. The lesions, which resembled bacterial speck lesions, probably resulted from injury received during germination and were not caused by any pathogen.

(ii) Germination on blotters in sealed plastic containers

Because the pathogen was not isolated when seeds were germinated in the Copenhagen tanks seeds were germinated on moist blotters, in sealed plastic containers, in temperature controlled germinators in the hope that the environmental conditions which favour development of the pathogen could be synthesised. The seeds were germinated at either 20C or 25C constant because temperatures in this range favour the development of the disease in the field (Basu 1966, Grogan *et al* 1974). The seed was germinated at 20-30C because these temperatures favour the germination of tomato seed and are the temperatures used in germination tests (International Seed Testing Association Rules).

Four trials involving the use of the sealed plastic containers for seed germination were set up.

TRIAL 1

Materials and Methods

Seven treatments were used as is outlined. All 'Beefsteak' seed used was hand extracted from diseased fruits with over 50 lesions; *P.syringae* pv *tomato* was isolated from 15.9% of the seeds. All 'VF 145-B-7879' seed used had been vacuum infiltrated in the usual manner. To avoid the introduction of any ubiquitous bacterial pathogens all seed was sown to moist, ultra-violet light irradiated blotters in plastic containers in a laminar flow cabinet.

Treatment 1

Two hundred 'Beefsteak' seeds were sown 50 per blotter, in sealed plastic containers and placed in the dark in a 15C germinator for 12 days at which time 100 germinating seeds were transferred to a 5C germinator for 15h and then placed in a 10-14h, 20-30C dark-light germinator. The remaining 100 seedlings were transferred directly to the 20-30C germinator.

Treatment 2

Four hundred 'Beefsteak' seeds were sown, 50 per blotter, in the dark in a 20C germinator. Two hundred seeds were maintained at 20C for the duration of the experiment. One hundred seedlings were placed in each of the 5C and 15C germinators in the dark for 15h soon after radicle emergence and then returned to the 20C germinator.

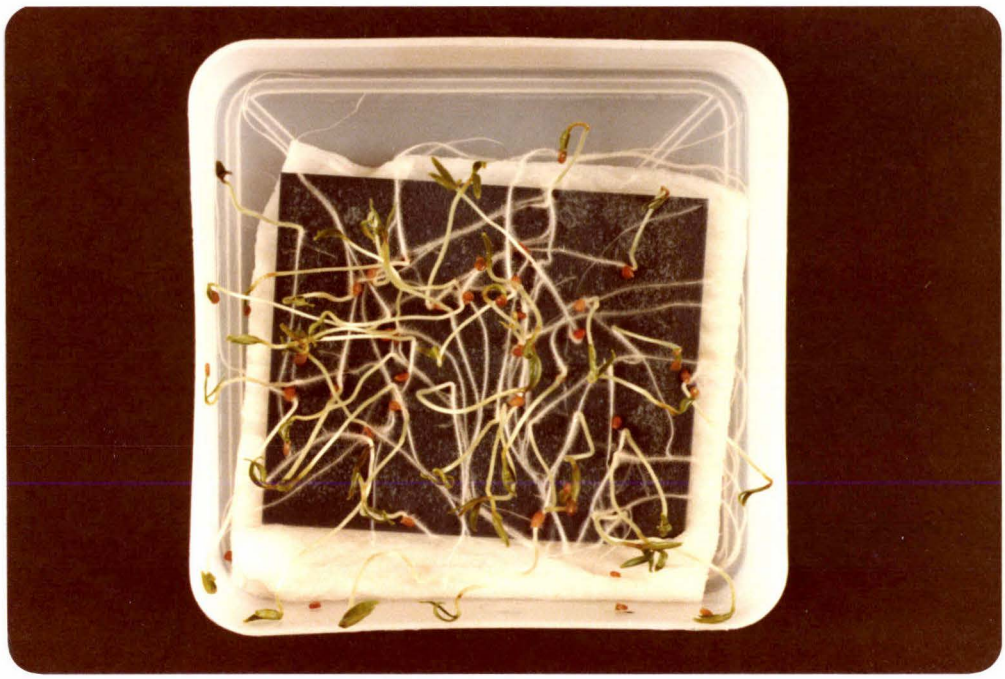


Plate 12: Germination of tomato seeds on moist blotters in sealed plastic containers

Treatment 3

Four hundred 'Beefsteak' seeds were sown to blotters in the 20-30C, 10-14h, dark-light germinator. Two hundred seeds were maintained in these conditions for the duration of the experiment. At radicle emergence 100 seedlings were placed in the 5C and 15C germinators for 15h in the dark and returned to the 20-30C germinator.

Treatment 4

Two hundred 'Beefsteak' seeds were sown in the sealed plastic containers and germinated at 25C in the dark. At radicle emergence 100 seedlings were placed in the dark at 15C for 15h and then returned to the 25C germinator containing the other 100 germinating seeds.

Treatment 5

Two hundred 'VF 145-B-7879' seeds were germinated in the dark at 25C. One hundred seedlings were exposed to 15C for 15h in the dark at radicle emergence and then returned to the 25C germinator containing the other germinating seeds.

Treatment 6

Two hundred 'VF 145-B-7879' seeds were germinated in the light at 30C. At radicle emergence 100 seedlings were incubated at 15C for 15h in the dark and then returned to the 30C germinator, where the other 100 seeds were germinated.

Treatment 7

Two hundred 'VF 145-B-7879' seeds were germinated in the 20-30C 10-14h dark-light germinator until radicle emergence when half the seedlings were transferred to a 15C, dark germinator for 15h and then returned to the 20-30C germinator containing the other 100 seeds.

All sealed plastic boxes were opened, only in the laminar flow cabinet. Any lesioned cotyledons or first leaves were detached from the seedlings, gently macerated in sterile water and then streaked to King's B medium and D4 agar. All colonies on D4 agar with a colony morphology similar to *P.syringae* pv *tomato* were streaked to King's B medium agar. All blue fluorescent colonies were tested for oxidase production with oxidase negative colonies being tested for pathogenicity to tomatoes.

Results and Discussion

Only seedlings with lesions which resembled bacterial speck were sampled. The pathogen was only isolated from one lesioned cotyledon and three lesioned first leaves (Table 16).

Table 16: Isolation of *P.syringae* pv *tomato* from lesioned cotyledons and first leaves of tomato seedlings germinated from infested seedlines.

Treatment*	Total No. of seeds germinated	No. of cotyledons & first leaves sampled	No. of Plates with blue fluorescent isolates	Oxidase reaction	Pathogenicity test
1	100	17	12	12+	
1 + 5C	100	18	6	6+	
2	200	8	6	6+	
2 + 5C	100	0			
2 + 15C	100	6	1	-	-
3	200	14	9	-	+
3 + 5C	100	5	0		
3 + 15C	100	6	5	-	-
4	100	24	7	-	-
4 + 15C	100	16	9	9+	
5	100	6	2	2+	
5 + 5C	100	4	3	-	-
6	100	12	4	-	-
6 + 15C	100	11	6	6+	
7	100	12	4	4-	3+
7 + 15C	100	4	2	-	-

* Details of each treatment are in the Materials and Methods

Most of the lesions are probably due to injuries incurred during the emergence of the cotyledons in epigeal germination.

A low temperature treatment was used in this trial because it was suggested by Roberts and Boothroyd (1972) that a low or high temperature can predispose some plants to pathogen attack. A low temperature treatment was used because tomatoes are susceptible to cool temperatures and exposure to these temperatures may reduce the resistance of the seedling to pathogen attack.

Isolations were made from whole cotyledons or first leaves and not only from the lesioned area. Therefore it was not established whether the lesions were caused by *P. syringae* pv *tomato*. The bacterial pathogen may have been present on the seedlings as resident populations (Schneider and Grogan 1977a) and the lesions could have resulted from injury and not be caused by a pathogen.

It was expected that the cool temperature treatment would increase the susceptibility of the seedling to pathogen attack. However, the pathogen was not isolated from any seedlings exposed to cool temperature treatments. Because it was isolated from so few seedlings germinated at 20-30C it is impossible to draw any conclusions concerning the effect of cold temperature treatment on the susceptibility of tomato seedlings to *P. syringae* pv *tomato*.

This work has demonstrated that seed-plant transfer can occur in both artificially infested and naturally infested, hand-extracted seedlines. Thus, *P. syringae* pv *tomato* can be a seedborne pathogen in naturally infested, hand extracted seed and, presumably, can also be seedborne in naturally infested commercial seedlines.

TRIAL 2

Having established that seed-plant transfer can occur the effect of different environmental conditions on seed-plant transfer was further investigated.

Materials and Methods

Vacuum infiltrated 'VF 145-B-7879' seed was sown to moist, ultra-violet light irradiated blotters in sealed plastic containers in the laminar flow cabinet. The seeds were germinated in seed germinators at 20C constant, 25C constant and 20-30C,10-14h. Either 100 or 200 seeds were used in each of the twelve treatments in all 3 germinators.

Treatment 1

The seed was germinated at all the temperature treatments in the dark.

Treatment 2

The seed was germinated in the 20C and 25C germinators in continuous light and in the 20-30C germinator with 10h dark, 14h light.

Treatment 3

The seed was germinated in the dark at each temperature treatment and the cotyledons were injured, soon after expansion, by gently brushing or squeezing the developing cotyledons with a light pair of forceps. The cotyledons were injured because injuring leaf trichomes is reported to favour bacterial speck infection (Schneider & Grogan 1977b).

Treatment 4

The seed was germinated in continuous light at 20C and 25C and in 14h (daylength) light at 20-30C and the cotyledons were injured in the same way as is described above.

Treatment 5

The seed was germinated in the dark at each temperature treatment. Soon after cotyledon expansion the seedlings were exposed to 10C temperatures for 12h in the dark and returned to the appropriate germinator.

Treatment 6

The seed was germinated in continuous light at 20 and 25C and day length light at 20-30C, exposed to 10C temperatures for 12h in the light, soon after cotyledon expansion, and returned to the appropriate germinator.

Treatment 7

The seed was germinated in the dark for each temperature treatment and, soon after expansion of the cotyledons, the seedlings were incubated at 35C for 12h in the dark and then returned to the appropriate germinator.

Treatment 8

The seed was germinated at 20C, 25C in the light and 14h light at 20-30C. Soon after the expansion of the cotyledons the seedlings were exposed to 35C for 12h in the light and returned to the appropriate germinator.

Treatment 9

Seed was germinated in the dark for each temperature treatment and, soon after the expansion of the cotyledons, the seedlings were incubated at 10C for 12h in the dark. Prior to this incubation, the cotyledons were gently injured as described above. After incubation all seedlings were returned to the appropriate germinators.

Treatment 10

Seed was germinated in continuous or daylength light until soon after the expansion of the cotyledons at which time seedlings at each temperature treatment were gently injured and incubated at 10C for 12h in the light. After this treatment seedlings were returned to the appropriate germinators.

Treatment 11

Dark germinated seedlings at each temperature treatment were gently injured soon after cotyledon expansion, exposed to 35C for 12h in the dark and returned to the appropriate temperature.

Treatment 12

Seed was germinated in continuous or daylength light. Shortly after cotyledon expansion the seedlings were gently injured, incubated at 35C for 12h in the light and returned to the appropriate germinators.

All sealed plastic containers were only opened in the laminar flow cabinet and all seedling parts were sampled 4 or 5 days after treatment either by macerating all seedling parts in sterile water and streaking to King's B medium agar and D4 agar or, by introducing the seedling parts to D4 broth, made more selective by the addition of 2.5 g/l of lithium chloride. The D4 broth was enriched by incubating for 16-30h on an orbital shaker at 80rpm at room temperature and then streaked to King's B medium agar or dilution plated to D4 agar. All colonies on D4 agar with morphology similar to *P. syringae* pv *tomato* were subcultured to King's B medium agar and all blue fluorescent colonies on this agar were tested for oxidase production, with oxidase negative colonies being tested for pathogenicity to tomatoes.

Results and Discussion

P.syringae pv *tomato* was only isolated from seedlings originating from the vacuum infiltrated seedline germinated at 20-30C (Table 17). Furthermore the pathogen was only isolated from those seedlings exposed to a sudden temperature increase or decrease other than the night day fluctuations. None of the seedlings, from which the pathogen was isolated, showed any bacterial speck like symptoms after 21 days. The pathogen was isolated from both the roots and shoots of the seedlings, but it was not regularly isolated from the cotyledons and first leaves. *P.syringae* pv *tomato* was isolated from seedlings germinated in complete darkness and those germinated in 14h light 10h dark but it was more frequently isolated from those seedlings germinated in the day length light.

Bashan *et al* (1978) reported that infection of tomato plants is favoured by high temperatures up to 35-40C and by free water accumulating on the leaves. High temperatures significantly increased disease incidence probably because the stomata open and the pathogen can infect through the stomata. Similarly they reported that cold temperatures result in stomatal closure and reduced infection. Although cool temperatures are reported not to favour infection, Schneider and Grogan (1977a) reported that the survival of resident populations was favoured by cooler temperatures, the population actually decreasing as temperatures increase from 17-32C when leaves are dry. If the leaves were misted for 24h or 48h, after inoculation resident populations increased most rapidly at 25C. At 32C the resident population decreased regardless of leaf wetness.

Table 17: Isolation of *P.syringae* pv *tomato* from tomato seedlings germinated from vacuum infiltrated 'VF 145-B-7879' seed

Germinator temp. and treatment *		No. of seeds germinated	No. of seedlings sampled	No. of plates with blue fluorescent colonies	Oxidase reaction	Pathogenicity test
20C	1	100	30	0		
	2	200	80	2	2+	
	3	100	50	2	2+	
	4	200	80	1	+	
	5	100	50	5	5+	
	6	200	80	1	+	
	7	100	50	0		
	8	200	80	10	10+	
	9	100	50	0		
	10	200	80	1	+	
	11	100	50	1	+	
	12	200	80	0		
25C	1	200	80	1	+	
	2	200	100	2	2+	
	3	200	80	0		
	4	200	100	1	+	
	5	200	80	0		
	6	200	100	0		
	7	200	100	1	+	
	8	200	100	3	3+	
	9	200	100	2	2+	
	10	200	100	0		
	11	200	100	0		
	12	200	100	1	+	
20-30C	1	200	100	0		
	2	200	150	6	6+	
	3	200	100	3	3+	
	4	200	150	0		
	5	200	100	2	2-	2+
	6	200	150	12	12-	12+
	7	200	100	2	2+	
	8	200	150	17	17-	17+
	9	200	100	1	-	+
	10	200	150	0		
	11	200	100	0		
	12	200	150	0		

* See materials and methods for details

Exposure to the high temperature, 35C for 12h, should result in an increase in the percentage of seedlings becoming infected because the pathogen invaded through the open stomata (Bashan *et al* 1978). This may explain why the pathogen was isolated from seedlings exposed to a high temperature treatment and not from those seedlings maintained at 20-30C for the duration of germination. *P.syringae* pv *tomato* was isolated from seedlings exposed to the cool temperature (10C for 12h) possibly because the cool temperature favoured the development of resident populations which could infect the seedlings when they were returned to 20-30C.

More successful isolations were made from seedlings germinated in day length light than those germinated in the dark, possibly because the increased stomatal opening in photosynthesis favoured infection by *P.syringae* pv *tomato*.

Schneider and Grogan (1977a) reported that injuring the basal cells of the leaf trichomes, followed by inoculation with *P.syringae* pv *tomato*, resulted in more lesion production than in the non-injured controls. Therefore, it was expected that injury alone or injury in combination with other predisposition treatments would increase the susceptibility of the seedlings to infection by *P.syringae* pv *tomato*. The pathogen was not isolated from any injured seedlings. Therefore the pathogen was either not associated with the seedlings or was not isolated from infested seedlings.

It is not known why the pathogen was only isolated from seedlings germinated at 20-30C. However, the pathogen may be associated with these seedlings because they develop rapidly at 20-30C and consequently have poorly developed protection mechanisms.

TRIAL 3

Materials and Methods

Some of the seedlings germinated at 20-30C with 14h light and exposed to either a high or low temperature fluctuation were transplanted to sterile soil at the early first leaf stage. The aim of this was to keep these seedlings growing for a sufficient period of time for symptom production to occur.

Unfortunately very few seedlings survived transplanting. However, the 19 surviving seedlings were incubated in the mist chamber for 2 weeks, at which time the foliage of each seedling was excised, macerated in sterile water and streaked to King's B medium agar. All blue fluorescent colonies were tested for oxidase production with any oxidase negative colonies being tested for pathogenicity on tomatoes.

Results and Discussion

Lesions developed on a number of the seedlings which survived the transplanting from blotters to the soil but *P. syringae* pv *tomato* was not isolated from any of the seedling parts sampled. Most of the lesions probably resulted from injuries received during transplanting. If more seedlings had survived the transplanting then the possiblity of seedling infection occurring or of a positive isolation being made would be increased. To few seedlings were used in this trial to draw any conclusions.

TRIAL 4

This trial aimed to isolate the pathogen from seedlings germinated from artificially and naturally infested seed and to establish how the pathogen was associated with the seedling after seed-plant transfer by surface sterilising the cotyledons and first leaves prior to isolating.

Materials and Methods

Vacuum infiltrated 'VF 145-B-7879' seed and 'Beefsteak' and 'Scoresby' seed, hand extracted from fruits with over 50 lesions, were sown to blotters and incubated at 20-30C in sealed plastic containers. Sixteen percent of the seeds in the 'Beefsteak' seedline were naturally infested with the pathogen but the pathogen was not isolated from the 'Scoresby' seedline (see 3.2.2). One hundred and fifty seeds of each seedline were germinated in each of three treatments:

Treatment 1

20-30C 10h dark 14h light for the duration of the experiment.

Treatment 2

20-30C 10h dark 14h light plus exposure to 35C for 12h in the light soon after cotyledon expansion.

Treatment 3

20-30C 10h dark - 14h light plus 12h at 10C, in the light soon after cotyledon expansion.

All seedlings were sampled 10 days after the temperature treatments either by macerating the seedling in sterile water, or by enrichment culturing the seedlings in D4 + LiCl broth for 16-30h. The macerated leaf or the enrichment culture were streaked to King's B medium agar and blue fluorescent isolates were tested for oxidase production with oxidase negative colonies being tested for pathogenicity to tomatoes.

Some of the seedlings, of each seedline, at each temperature treatment were surface sterilised in 1.0% sodium hypochlorite for five minutes, rinsed with sterile water and then macerated or

enrichment cultured, streaked to King's B medium and tested for the pathogen in the usual manner. Surface sterilising the seedlings should inactivate any of the bacteria not infecting the seedlings or not in a protected position (e.g. leaf trichomes) on the seedlings and should give some indication of how the pathogen is associated with the seedling if seed-plant transfer occurs.

Results and Discussion

A number of lesions developed on the germinating seedlings but none of these resembled bacterial speck symptoms. A large number of seedlings were sampled regardless of the lack of symptoms. *P.syringae* pv *tomato* was isolated from symptomless 'Beefsteak' seedlings germinated at 20-30C with daylength light and exposed to 10C for 12h in the light. The pathogen was not isolated from the seedlings of the other varieties (Table 18) and it was not isolated from any of the 'Beefsteak' seedlings which were surface sterilised prior to isolation.

The pathogen was not isolated from any seedlings germinated from the vacuum infiltrated seedline either, possibly because the seed was not infested, or because insufficient seeds were germinated to ensure the association of the pathogen with the seedlings as a result of seed-plant transfer.

Schneider and Grogan (1977a) reported that the pathogen survived as resident populations on the tomato leaves. Surface sterilisation would inactivate most resident populations on the seedlings. Thus, if the pathogen had been isolated after surface sterilisation it would indicate that *P.syringae* pv *tomato* had probably infected the seedlings and was present in the incubation phase or as a latent infection. Unfortunately, the supply of naturally infested seedlines used in this trial was limited thus restricting the size of the trial to such an extent that no conclusions could be drawn.

Table 18: Isolation of *P.syringae* pv *tomato* from tomato seedlings germinated at 20-30C

Treatment *	No. of seeds germinated	No. of seedlings sampled	No. of plates with blue fluorescent colonies	Oxidase reaction	Pathogenicity test
VF 145	100	50	0		
VF 145 + cold	100	80	1	+	
VF 145 + warm	100	80	0		
VF 145 SS+[2]	50	20	0		
VF 145 + cold SS+	50	30	0		
VF 145 + warm SS+	50	30	0		
'Beefsteak'	100	50	5	5+	
'Beefsteak' + cold	100	80	2	2-	2+
'Beefsteak' + warm	100	80	3	3+	
'Beefsteak' SS+	80	80	0		
'Beefsteak' cold SS+	80	80	1	-	-
'Beefsteak' warm SS+	80	80	0		
'Scoresby'	100	50	2	2+	
'Scoresby' + cold	100	80	0		
'Scoresby' + warm	100	80	1	+	
'Scoresby' SS+	50	20	0		
'Scoresby' + cold SS+	50	20	0		
'Scoresby' + warm SS+	50	20	0		

* See Materials and Methods for details on treatments

[1]VF 145 = 'VF 145-B-7879'

[2]SS+ = Surface sterilised with 1.0% sodium hypochlorite

(iii) Germination in a mist chamber

Materials and Methods

As well as performing germination tests on blotters, vacuum infiltrated 'VF 145-B-7879' seed, hand extracted 'Beefsteak' seed (16% infected) and a commercial 'Moneymaker' seedline (at least 0.14 percent infested) were germinated in sterile soil in a mist chamber at 16-25C. The water supply, used in the mist chamber, was tested for the presence of the pathogen prior to the commencement of this work. No blue fluorescent isolates were apparent on King's B medium agar after 4 days incubation. The only possible source of inoculum, besides the seed, is any ubiquitous speck organisms present in the air or on the mist chamber walls. As a control, pathogen free seed was germinated in sterile soil, in isolation from infested seeds, in the mist chamber.

Seedling parts were sampled soon after emergence, at the first leaf stage and at least one month after sowing by placing seedlings in D4 broth plus LiCl. All root samples were washed in sterile water prior to enrichment culturing. The broth was enriched for 16-30h and then streaked to King's B medium agar for 1-2 days at 25C. Blue fluorescent colonies were tested for oxidase production with oxidase negative colonies being tested for pathogenicity to tomatoes.

Results and Discussion

The pathogen was not isolated from any of the seedlings, germinated from either the vacuum infiltrated 'VF 145-B-7879' seedline or the naturally infested hand extracted 'Beefsteak' seedline, in the mist chamber. Some of the 'Moneymaker' seedlings germinated from a bacterial speck infested commercial seedline, developed lesions typical of bacterial speck. However, attempts to isolate from these lesioned seedlings were unsuccessful.

Although Bashan *et al* (1978) and Kim (1979) were able to isolate *P. syringae* pv *tomato* from seedlings, germinated from naturally infested seedlines in moist chambers, the pathogen was not isolated from any seedlings germinated in the mist chamber in this study. All seedlines were infested with the pathogen so why seed-plant transfer did not occur can not be explained. The environmental conditions may have differed although Kim (1979) reported that seedlings germinated at similar temperatures in a moist chamber, became infected.

(iv) Germination in commercial nurseries

Materials and Methods

The germination of seedlings in two commercial nurseries were observed in an attempt to identify seed-plant transfer in a commercial situation. The time when infection first appeared in each nursery was recorded and correlated with the environmental conditions prior to the disease outbreak. Department of Scientific and Industrial Research, Palmerston North's meterological readings were used to record outside temperatures and rainfall and Ohakea Air Force Base's Aero Weather Report was used for all barometric pressure readings and relative humidity records.

In one nursery seeds of 5 varieties, viz., 'Beefsteak', 'Grosse Lisse', 'Moneymaker', 'Russian Red', and 'Scoresby' were germinated in methyl bromide treated soil (Plate 13) and the seedlings were observed, at least once a week, from emergence until sale between 6 August 1979 (6.8.79) and 31 October 1979 (31.10.79). The temperature was recorded in the glasshouse on a thermograph with a range from -30C to 40C. In the other nursery, seedlings of the varieties 'Moneymaker', 'Potentate', 'Russian Red', and 'Scoresby' were observed at least once a week from 6.8.79 to 31.10.79 between germination and sale. The temperature was not monitored at this nursery. All seedlings in both nurseries were overhead watered.

In this work there was no control over outside sources of inoculum. The pathogen may have been present in the treated soil, in the unsterilised seedling trays, on the unsterilised glasshouse walls or floors. If the pathogen is ubiquitous, it may even be airborne. The water supply was sampled for the pathogen and *P.syringae* pv *tomato* was not isolated. As a partial control each seedline was germinated in sterile soil, in sterile seedling trays in an unheated University glasshouse, which had no past record of tomato growing. The seedlings were transplanted as in the nursery situation and returned to the nursery for sale. Only one sowing was made for each seedline in early September 1979. The temperature in this glasshouse was monitored using a thermograph.

Results and Discussion

No seedlings in the commercial nurseries or in the control glasshouse showed any speck symptoms during August or September 1979. Seedling infection occurred in the two commercial nurseries between 2.10.79 and 8.10.79. Bacterial speck was first observed in 'Beefsteak' seedlings, in the cold frames, in one nursery. In the other nursery 'Moneymaker' was the first variety in which the disease was evident with both seedlings in the glasshouse and the cold frames showing speck symptoms. The incidence of disease was much higher in the cold frames than in the glasshouse, but in one corner of the glasshouse under a leak in the roof a high percentage of the seedlings were infested.

Initially the disease was only present in these two varieties and the incidence of disease in these varieties increased rapidly. Some of the other varieties grown in the nurseries, viz. 'Scoresby' and 'Potentate' are very susceptible to bacterial speck. However, the incidence of disease was not high in any other varieties and no symptoms appeared in these varieties until after the 15.10.79 suggesting they were not exposed to the source of primary inoculum.

Plate 13: Tomato seedlings in a glasshouse at a commercial nursery.

This work did not isolate the seedlings from all other sources of inoculum. All varieties were grown under the same conditions and the disease was only prevalent in 'Beefsteak' and 'Moneymaker' seedlings, although all varieties are susceptible to bacterial speck. This indicates that the pathogen probably infected these seedlings from a source of inoculum to which only they were exposed. The only possible source of inoculum, not common to all seedlings in each nursery, is the seed. The fact that *P. syringae* pv *tomato* was isolated from the 'Beefsteak' and 'Moneymaker' seed by use of the Cooke's microtitre plate system and the agar plug technique further indicates that the seed is likely to be the source of primary inoculum.

Although the pathogen probably infected the seedlings as a result of its association with the seed, this does not explain why the disease first appeared in the seedlings between 2.10.79 and 8.10.79 when both seedlines were germinated in the nursery and the University glasshouse between 6.8.79 and 31.10.79. The environmental conditions between the 21.8.79 and 20.10.79 were studied to determine if there was any correlation between the environmental conditions and the time of disease outbreak. Some relationship between these two factors does appear to exist, hence a more detailed explanation is presented.

In the nursery where the thermograph recordings were made the disease was only apparent in cold frames. Accordingly only the outside maximum and minimum temperatures as recorded by the DSIR, Palmerston North (Appendix IV, Figure 2) were compared with the time of disease outbreak. Because of a microclimate effect the temperature in the cold frames in the vicinity of the plants is probably 1-2C higher than the temperature shown in the histogram. The mean daily relative humidity was calculated from the recordings made at Ohakea Air Force Base because the DSIR only makes one relative humidity reading a day (Appendix IV, Figure 3). This figure can only be taken as a guide because Ohakea is some 30 km from Palmerston North and because of the boundary layer effect on relative humidity caused by transpiring leaves (Schneider and Grogan 1977a). The daily rainfall

(Appendix IV, Figure 3) recorded at DSIR, Palmerston North, was only graphed after the 11.9.79 because prior to this date any seedlings, which later showed symptoms of bacterial speck, were in the glasshouse and were overhead watered daily.

Strobel and Mathre (1970) report that a change from high to low barometric pressure increased the susceptibility of beans to a *Pseudomonas* species and the susceptibility of tobacco to *P. syringae* pv *tabaci*. A study of the barometric pressure (Appendix IV, Figure 4) and relative humidity histograms shows that the barometric pressure is approximately an inverse measure of relative humidity. The author suggests that the increased susceptibility of beans and tobacco to pseudomonad phytopathogens reported by Strobel and Mathre (1970) is due to an increase in relative humidity and not to the decreased barometric pressure.

The maximum and minimum temperatures, the mean daily relative humidity and the rainfall were used to identify periods when the environmental conditions would favour bacterial speck development in the 7 weeks preceeding the disease outbreak. Basu (1966) reported that distinct bacterial speck symptoms developed when conditions of high humidity (87-97%) and moderate temperature 23-28C prevailed for 5-14 days. Schneider and Grogan (1977a) reported that free moisture on the leaf promotes growth of resident populations and infection. Other researchers have also reported that high relative humidities, temperatures in the 17-26C range and free leaf moisture favour infection (Grogan *et al* 1974; Schneider and Grogan 1977 a and b; Bashan *et al* 1978 and Pohronezny *et al* 1979).

In this research all daily mean relative humidities over 85% were identified and compared with the temperature and rainfall. The relative humidity averaged over 85% on 5 days between the 21.8.79 and the 11.9.79. On all high relative humidity days, except for 2.9.79, the maximum and minimum temperatures were below 15C and 10C respectively, temperatures which have not been reported to favour bacterial speck development. On 2.9.79 the average relative humidity

was 89% and the temperature reached a maximum of 17C and a minimum of 11.5C. If the effect of the leaf microclimate is taken into consideration, then it is possible that these environmental conditions could favour bacterial speck development. Between 12.9.79 and 16.9.79 the rainfall kept the leaf surfaces of any plants in the cold frames moist and the temperature and relative humidity were favourable for speck development. By examining the histograms it appears that the environmental conditions were also favourable for speck development on 29.9.79 and 30.9.79 and again on 8.10.79 and 9.10.79.

The disease outbreak may have originated from the favourable environmental conditions on 2.9.79 or on 12.9.79 - 16.9.79. At this time *P.syringae* pv *tomato* could have become established as resident populations on the leaves (Schneider and Grogan 1977a) and survived as resident populations until 29.9.79 or 30.9.79 when the conditions favoured both infection and spread by rainsplash. Bacterial speck symptoms were apparent 8 days later when the environmental conditions again favoured spread and development of the pathogen. An increase in the number of infected seedlings was observed between 8.10.79 and 15.10.79, possibly resulting from the favourable conditions for bacterial speck development on 8.10.79 and 9.10.79.

Although any relationship between environmental conditions and disease outbreak may be purely circumstantial, a correlation between the two does appear to exist and provides an explanation for the appearance of bacterial speck symptoms on 8.10.79. However, to extract any conclusive evidence from such data, a large number of nurseries would need to be surveyed for a number of years.

3.4.5 Conclusion

When this research commenced, there was no conclusive evidence that *P. syringae* pv *tomato* could be seedborne. However, since commencing this study, work by Bashan *et al* (1978) and Kim (1979) has presented evidence that *P. syringae* pv *tomato* can be seedborne. The results presented herein substantiate these reports and prove that *P. syringae* pv *tomato* can be seedborne in New Zealand. The question of the seedborne nature of the pathogen was approached in a stepwise manner. *P. syringae* pv *tomato* fulfilled the first two requirements tested for it to be seedborne, viz, plant-seed transfer and seed-seed transfer and although it is not possible to conclusively report that the pathogen can infect the seedlings causing bacterial speck symptoms, it is possible to conclude that seed-plant transfer can occur. The appearance of bacterial speck lesions on seedlings in a commercial nursery appears to be related to the use of infested seed, thus demonstrating that symptoms are likely to result from seed-plant transfer. However, other sources of inoculum can not be excluded.

The importance of seedborne *P. syringae* pv *tomato* has not been reported in the literature, but, its importance can be speculated upon. Obviously the association of the pathogen with the seed enables it to be transported to new areas and, because it can be seedborne, it can be the source of primary inoculum in these previously bacterial speck free areas. The association of *P. syringae* pv *tomato* with the seed enables it to be constantly associated with a susceptible host and thus, it could survive between seasons and infect the seedlings soon after emergence. These early infections can be responsible for large losses in the nursery, expedite the onset of an epidemic and be responsible for the yield losses which are reported to result from early infections, although the plants may appear to recover (Grogan *et al* 1974).

It was not possible to determine how the pathogen infests the seedling as a result of its association with the seed, and although the effect of environmental conditions on seed-plant transfer was discussed, no conclusions concerning this can be drawn.

Because the seedborne nature of *P.syringae* pv *tomato* may be important as a source of primary inoculum, the use of seed treatments to control such inoculum was investigated.

3.5 Seed Treatments

Work by Bashan *et al* (1978), Kim (1979) and earlier work in this study has established that *P.syringae* pv *tomato* can be seedborne. The importance of *P.syringae* pv *tomato* being seedborne has been speculated on in the conclusions on seed-plant transfer.

If the pathogen is widespread in its occurrence it could be introduced into new areas or initiate infections in the nursery even if all of the speck organisms in association with the seed are inactivated. For these reasons it may seem pointless using clean seed, as the pathogen could infect the plants any way. However, in the nursery, a speck epidemic is likely to occur early if the pathogen is in association with the seed and infects seedlings soon after germination. If *P.syringae* pv *tomato* infects the seedling, as a result of its ubiquitous nature, then the chance association and infection, is more likely to occur in established seedlings with larger leaf surface areas for the pathogen to be deposited upon. The use of clean seed would, at the least, delay the epidemic or could result in the production of healthy seedlings if no other sources of the bacteria are present. Because nurserymen cannot sell diseased plants, economic loss may result from the dumping of large numbers of diseased seedlings. Growers of tomato transplants may suffer economic losses as indicated by the 13 percent yield reductions reported to occur in plants which appeared to recover from early bacterial speck infections (Grogan *et al* 1974). For these reasons it may be economic for a nurseryman to use clean seed and produce speck free seedlings. However, if *P.syringae* pv *tomato* free seed is used the grower would also have to endeavour to control any other sources of inoculum.

Because it only needs one infested seed and in turn one diseased seedling to cause an epidemic, any clean or treated seed has to be completely pathogen free. The production of bacterial speck free tomato seed crops is one way of producing pathogen free seed. Another approach is to treat the seed in some way which completely controls any *P.syringae* pv *tomato* in association with the seed.

a. Extraction

If a seed treatment is available which will give complete control of the pathogen, ideally, it should be incorporated into a seed extraction or cleaning programme. Several seed extraction procedures exist, all of which may directly influence the survival of the pathogen.

(i) Uncleaned seed

The ripe fruits are cut open and the pulp is squeezed out, dried on paper at room temperature and the seed is packaged. The only way the pathogen can be inactivated is by desiccation.

(ii) Fermentation

Well ripened fruits are cut, the pulp is squeezed into containers and placed in a warm place until the seed floats to the surface, separating it from the seed sacs (Chamberlain and Fry, 1950). Fermented pulp containing *P. syringae* pv *tomato* was infected after 24h but not after 48h (Chambers and Merriman, 1975).

(iii) Acidification

The pulp of ripe fruit is mixed with commercial hydrochloric acid (HCl) for 3h and the seed washed and dried (Chamberlain and Fry, 1950). Most tomato seed is extracted by the use of HCl which controls any tobacco mosaic virus present as a seed contaminant and leaves a clean, bright seed after extraction. Chambers and Merriman (1975) reported that the bacterial pathogen, *P. syringae* pv *tomato* was unable to survive in acid-extracted pulp.

Although acid-extraction might prevent the survival of the pathogen in association with the seed, the use of other treatments was also considered.

b. Other germicidal treatments

A number of chemicals have been reported to have bacterial activity against *Corynebacterium michiganense* pv *michiganense* in tomato seed. Ark (1944) reported that alcohol and a number of dyes were successfully used to control *C.michiganense* pv *michiganense* on the seed without reducing the seed germinability or seedling vigour. Shoemaker and Echandi (1976) found that a substantial reduction of bacterial canker in seedlings can be obtained by treating the seed with either dilute hydrochloric acid (5%) or dilute sodium hypochlorite (1.05%). More dilute sodium hypochlorite (0.6%) is also reported to control the bacterial canker organism (Kimble pers. comm. 1980) and may be useful as a seed surface sterilant to control *P.syringae* pv *tomato*.

Bashan *et al* (1978) reported that a 'surface sterilisation' process, which involved exposing the seed to 70% ethanol followed by 1.0% sodium hypochlorite (each for 30 seconds under vacuum) and five washes with sterile water, resulted in all tomato seeds becoming free of *P.syringae* pv *tomato* . The author herein questions the claim that this practice involves surface sterilisation but rather considers it to be an infiltration process which would not only inactivate surface contaminants but also could inactivate any bacteria infecting the seed.

Because it was not possible to artificially inoculate Spergon^{R} (tetrachloroparabenzoquinone) treated 'Scoresby' seed with *P.syringae* pv *tomato*, Spergon^{R} may have potential as a bactericidal treatment. Because Spergon^{R} is no longer available in New Zealand so the germicidal activity of another quinone, Dichlone 50W^{R} (2-3, dichloro 1,4 napthoquinone), was investigated. Dichlone 50W^{R} is toxic to nitrogen fixing bacteria (Neergaard 1977) and also has some antibacterial activity against *Xanthomonas campestris* pv *vesicatoria* (Doidge) Young *et al* (Dye, 1969). Tate and van der Mespel (1976) and Tate (pers.comm. 1978) reported that Dichlone 50W^{R} gave inadequate control of *P.syringae* pv *tomato* on tomato foliage. There are no reports

of the use of Dichlone 50W[R] as a bactericidal seed treatment although Thomas (1959) used it as a seed treatment for control of damping off fungi.

c. Antibiotics

Antibiotic treatments have been successfully applied for the control of bacterial speck in the field (Grogan *et al* 1975, Tate and van der Mespel 1976). Streptomycin sulphate* has been used as a seed treatment to control a number of seedborne bacterial pathogens (Thomas 1959, Taylor and Dye, 1976). Seed can be treated by soaking, slurry treating or dusting with streptomycin sulphate (Taylor and Dye, 1976). Soaking pea seed in streptomycin sulphate solution at room temperature effectively controlled *Pseudomonas syringae* pv *pisi* (Sackett), Young, Dye and Wilkie, but it also reduced percentage germination. Slurry treating pea seed with streptomycin sulphate (2.5g/a.i. in 10ml of H_2O/kg of seed) gave good control of the pathogen without affecting seed germination whereas dusting (2.5g a.i. per kg seed) gave unacceptable control (Taylor and Dye, 1976). Accordingly, streptomycin sulphate applied in the correct form, could possibly provide an effective seed treatment to control *P.syringae* pv *tomato*.

d. Heat treatments

Heat treatments have been regularly used to control seedborne pathogens. Such treatments rely on the thermal inactivation point (TIP) of the pathogen being lower than the TIP of the seed. Hot water seed soaks, dry heat and steam-air treatments may all be useful in control of seedborne pathogens. Steam-air treatments have a number of advantages over hot water soaks (Baker, 1969) but are used relatively infrequently for control of seedborne pathogens.

Although *P.syringae* pv *tomato* has a TIP of approximately 50C (Bryan 1933), Chambers and Merriman, 1975), it can be inactivated by exposure to dry heat at 37C for 6 days. Temperatures above 50C

* Available in New Zealand as Agrimycin 17[R]

will inactivate the bacterium but they also reduce seed germination (Ark, 1944). Chambers and Merriman (1975) reported that no infected seedlings resulted from the germination of fermentation extracted seed, hot water treated at 55C for 25 minutes. No conclusions can be drawn from this data as bacterial speck did not develop on any of the seedlings germinated in their work.

The effectiveness of acid extraction, sodium hypochlorite, Dichlone 50W^{R} and streptomycin sulphate as seed treatments were evaluated in this study.

3.5.1 Acid extraction

Materials and Methods

The acid extraction process described by Chamberlain and Fry (1950) was used in this work. Commercial hydrochloric acid (28 ml) was added to the pulp of 2.5 kg of fruit. Pulp and acid were mixed well, stirred every half hour during the three hour treatment and then the seed was thoroughly washed with sterile water and dried.

Because all experimental work was conducted with seed already extracted from tomato fruits it was necessary to determine the amount of acid available to each individual seed in a pulp, so that seeds in the experimental situation would be exposed to the same concentration of acid as seeds in the pulp. The average percentage weight of seeds in tomato fruits was determined and the amount of HCl required to treat seed samples calculated. Approximately 2.5% of each fruits weight is seed; therefore commercial HCl would be added to a seed sample at 0.05 ml per gram of seed.

Seed of 'VF 145-B-7879' was vacuum infiltrated with a virulent culture of *P.syringae* pv *tomato* and acid extracted with 0.5 ml of one-tenth strength commercial HCl per gram of seed. The percentage of vacuum infiltrated seed infested with *P.syringae* pv *tomato* was determined, prior to acid extraction, by use of the Cooke's

microtitre plate system. The acid extracted seed was tested for the presence of the pathogen in two ways: (i) 50 whole seeds were added to 10 ml of King's B medium broth in each of 12 McCartney bottles and streaked to King's B medium agar after 24h at 25C: (ii) two grams of seed were ground in a grinder and the flour suspended in 10 ml of sterile water, shaken overnight on an orbital shaker at 80 rpm and streaked to King's B medium agar. All blue fluorescent colonies on King's B medium agar were tested for oxidase production with oxidase negative colonies being tested for pathogenicity to tomatoes. The percentage of vacuum infiltrated acid extracted seeds infested with the pathogen was determined by use of the Cooke's Microtitre Plate system.

The effect of acid extraction on a naturally infested 'Beefsteak' seedline and a 'Scoresby' seedline, both hand extracted from fruit with over 50 lesions, was investigated. An effort was also made to establish how the naturally infested commercial 'Beefsteak' and 'Moneymaker' seedlines were extracted.

Results and Discussion

P.syringae pv *tomato* was isolated from vacuum infiltrated 'VF 145-B-7879' seed after acid extraction when whole seeds were added to broth and then streaked to agar and when the ground seed suspension was streaked to agar (Table 19).

Table 19: Isolation of *P.syringae* pv *tomato* from vacuum infiltrated, acid extracted 'VF 145-B-7879' seed

Isolation method	No. of replicas	Blue fluorescent isolates	Oxidase reaction	Pathogenicity
Broth to agar	12	5	5-	5+
Ground seed suspension to agar.	8	8	8-	8+

The percentage of infested seeds was determined, both prior to and after acid extraction, to establish what percentage of infested seeds survived acid extraction (Table 20). Acid extraction reduced the percentage of artificially infested seeds in this trial by 97.3% and, in this artificial situation, did not completely control *P. syringae* pv *tomato*.

Table 20: Percentage of 'VF 145-B-7879' seeds infested with *P. syringae* pv *tomato* after vacuum infiltration and after vacuum infiltration plus acid extraction

Treatment	No. of seeds tested	No. of seeds infested	% of seeds infested
vacuum infiltrated	1152	637	55.3
vacuum infiltrated + acid extraction	960	15	1.5

P. syringae pv *tomato* was not isolated from any of the hand extracted, acid extracted 'Beefsteak' (Table 21) or 'Scoresby' seedlines. In this trial infestation of the naturally infested 'Beefsteak' seedline was eliminated. If acid extraction reduces the percentage of infested seeds in the 'Beefsteak' seedline to the same extent as it reduces the percentage of infested seeds in the artificially infested seedline, then a 97.3% reduction in percentage infestation is to be expected. Thus when the 'Beefsteak' seedline (15.9% infested prior to acid extraction) was acid extracted, only 0.5% or 5 seeds in the 1056 tested would be expected to be infested in this trial. The expected number of infested seeds in the sample is so small that it is possible that *P. syringae* pv *tomato* was not isolated because no infested seeds were present in the sample tested.

Table 21: Percentage of hand extracted 'Beefsteak' seeds naturally infested with *P.syringae* pv *tomato* before and after acid extraction

Treatment	No. of seeds tested	No. of seeds infested	% of seeds infested
hand extracted	1056	167	15.9
acid extracted	1056	0	0

It was not possible to establish how the commercial 'Beefsteak' seedline was extracted but the 'Moneymaker' seedline was acid extracted by a local nursery. The pathogen was isolated from 0.14% of the 1440 acid extracted seeds tested.

Acid extraction considerably reduced the percentage of seeds infested with *P.syringae* pv *tomato* in both the artificially infested and commercial seedlines. However, control was incomplete so acid extraction is inadequate as a seed treatment because plant infection could still result from the very low percentage of *P.syringae* pv *tomato* infested seeds surviving.

Although the acid extraction process does not give complete control it significantly reduced the percentage of infested seeds in both artificially infested and naturally infested seedlines. Therefore, because acid extraction controls seed contaminant TMV, produces bright clean seeds, and also reduces the percentage of seeds infested with *P.syringae* pv *tomato*, it is recommended that all tomato seed should be acid extracted. However, further seed treatment is necessary to guarantee complete control of *P.syringae* pv *tomato* associated with the seed.

3.5.2 Sodium Hypochlorite

Materials and Methods

One volume of vacuum infiltrated 'Dorchester' seed was added to 3 volumes of 0.3 or 0.6% a.i. sodium hypochlorite (Janola^{R}) for a 20, 40 or 60 min. soak. Seeds were rinsed thoroughly with sterile water and tested for the presence of the pathogen by use of the Cooke's microtitre plate system. Untreated vacuum infiltrated 'Dorchester' seed was tested as a control.

Results and Discussion

P. syringae pv *tomato* was isolated from the vacuum infiltrated sodium hypochlorite treated 'Dorchester' seed samples and from the untreated control samples (Table 22).

Table 22: Vacuum infiltrated 'Dorchester' seeds infested with *P. syringae* pv *tomato* both before and after sodium hypochlorite treatments

Treatment	No. of seeds tested	No. of seeds infested	% of seeds infested
Untreated	576	89	15.5
0.3% 20 min	672	20	3.0
0.3% 40 min	192	1	0.5
0.3% 60 min	192	5	2.6
0.6% 20 min	192	11	5.7

Although sodium hypochlorite treatments considerably reduced the percentage of infested seeds in a sample, control of the pathogen was incomplete. The maximum reduction in percentage infestation was 97% the same as for acid extraction, but the minimum reduction was only 66% with an average of 80% reduction in this trial.

Sodium hypochlorite treatments at both concentrations and at all time intervals tested failed to control the pathogen, thus making the treatment of little use as a seed treatment. The use of sodium hypochlorite at higher concentrations (e.g. 1.0%) were not tested but it is possible these may have given better control of any *P.syringae* pv *tomato* associated with the seed. Longer exposure times may result in reduced seed germination as a result of the greater imbibition of sodium hypochlorite.

Although the maximum reduction in percentage infestation is similar to that obtained with acid extraction the mean and minimum reductions are considerably lower. Because the reduction in percentage infestation is no better than for the acid extraction process, acid treatments, which are also useful for seed extraction, would be preferentially used as a seed treatment.

5.3.3 Dichlone 50W^{R}

Materials and Methods

Vacuum infiltrated 'Dorchester' seed was slurry treated with Dichlone 50W^{R} at 3.5 and 24.5 g per kg of seed. The slurry treatment involved mixing 100ml of water per kg of seed, with the appropriate amount of Dichlone 50W^{R}, and then drying the seed at room temperature. All 'Dorchester' seed used was vacuum infiltrated and the percentage infestation of both treated and untreated seed was determined by use of the Cookes' microtitre plate system.

Results and Discussion

P.syringae pv *tomato* was isolated from a lower percentage of seeds treated with Dichlone 50W^{R} at either 3.5 or 24.5 g/kg than from the untreated seeds. The pathogen was isolated from a similar percentage of seeds after both the 3.5 and 24.5 g/kg treatments (Table 23). The percentage reduction in infestation of 'Dorchester' seed as a result of the Dichlone 50W^{R} seed treatment was 81%.

Table 23: Infestation of vacuum infiltrated 'Dorchester' seed with *P.syringae* pv *tomato* both before and after Dichlone 50W^{R} seed treatments

Treatment g/kg seed	No. of seeds tested	No. of seeds infested	% of seeds infested
Untreated	576	188	32.0
3.5	440	30	6.8
24.5	384	23	6.0

Although Dichlone 50W^{R} seed treatments considerably reduced the percentage of seeds infested with *P.syringae* pv *tomato* control was incomplete. Because a seven-fold increase in the Dichlone 50W^{R} concentration did not significantly reduce the percentage of seeds infested it is unlikely that increasing the Dichlone 50W^{R} concentration further would reduce the number of seeds infested. Acid extraction and sodium hypochlorite treatments can be more effective in reducing the percentage of seeds infested with *P.syringae* pv *tomato*. Acid extraction is recommended in preference to a sodium hypochlorite or a Dichlone 50W^{R} seed treatment but a seed treatment giving complete control is required.

3.5.4 Streptomycin sulphate

Materials and Methods

Taylor and Dye (1976) reported that streptomycin sulphate used as a slurry treatment for pea seed was more satisfactory than either dusting or seed soaks. Accordingly, in this work only slurry treatments were tested. The rate used by Taylor and Dye (1976) of 2.5 g of streptomycin sulphate (745 units/mg) in 10ml H_2O/kg of seed was used initially. Half this rate, 1.25g streptomycin sulphate (745 units/mg)/kg of seed was also tested. Vacuum infiltrated 'Dorchester' seed was used and the percentage of infested seed in treated and untreated seed samples was determined using the Cooke's microtitre plate system.

The effect of streptomycin sulphate slurry treatments on seed germination and seedling vigour was investigated by germinating untreated seed and treated seed (2.5g/kg of seed) on moist blotters at 25C in a Copenhagen tank. The untreated 'Dorchester' seed was germinated as a control and samples of streptomycin sulphate treated 'Dorchester' seed were germinated weekly for 10 weeks after treatment. The percentage seed germination and seedling vigour were recorded 7 days after sowing.

Results and Discussion

The pathogen was not isolated from any streptomycin sulphate treated seeds but it was isolated from the untreated seeds (Table 24). No bacteria grew on King's B medium agar, streaked with King's B medium broth which had been inoculated with seed treated with streptomycin sulphate at 2.5 g/kg of seed. However, bacteria, other than *P. syringae* pv *tomato*, grew on agar streaked with an enriched culture of broth containing seeds treated with 1.25 g streptomycin sulphate per kg of seed.

Table 24: Infestation of vacuum infiltrated 'Dorchester' seeds with *P. syringae* pv *tomato* before and after streptomycin sulphate seed treatments

Treatment	No. of seeds tested	No. of seeds infested	% of seeds infested
Untreated	576	184	32
2.5 g/kg of seed	672	0	0
1.25 g/kg of seed	288	0	0

All *P. syringae* pv *tomato* in association with the seed was eliminated when seed was treated at both 2.5 and 1.25 g of streptomycin sulphate per kg of seed.

Because the streptomycin sulphate treatment gave complete control of *P.syringae* pv *tomato*, germination tests were performed to determine if streptomycin sulphate slurry treatments had a detrimental effect on seed germination or seedling vigour.

At the rates tested streptomycin sulphate treatment did not reduce the percentage seed germination but did appear to retard the speed of seed germination and reduce the vigour of a few seedlings after seed had been stored 6 weeks. This was even more noticeable from week 7 onwards (Table 25).

Table 25: Germination of 'Dorchester' seed treated with 2.5 g of streptomycin sulphate per kilogram of seed

Time (in weeks)	No. of seeds germinated	percentage germination	comments on seedling vigour (germination & growth)
Untreated control	200	98	even and rapid
treated 0	200	97	even and rapid
treated 1	100	98	even and rapid
treated 2	100	97	even and rapid
treated 3	100	96	even and rapid
treated 4	100	95	even and rapid
treated 5	100	95	even and rapid
treated 6	100	100	even and moderate
treated 7	100	99	even and moderate
treated 8	50	98	even and moderate
treated 9	50	96	uneven and slow
treated 10	45	93	uneven and slow

In this trial streptomycin sulphate gave complete control of any *P.syringae* pv *tomato* associated with the seed. However, some bacteria survived the 1.25 g of streptomycin sulphate per kg of seed and therefore, *P.syringae* pv *tomato* might also survive this treatment. To reduce the possibility of *P.syringae* pv *tomato* surviving and gaining

resistance to streptomycin sulphate seed treatments it is probably advisable to use the more concentrated streptomycin sulphate seed treatment.

The streptomycin sulphate treatment can not be used in the extraction and cleaning process because it is apparent from the trial that prolonged exposure to this chemical is likely to reduce seedling vigour. Streptomycin sulphate is best applied immediately prior to sowing to control any *P.syringae* pv *tomato* not controlled by acid extraction. Storage of streptomycin sulphate treated seed may be possible if the antibiotic application is followed by an 0.5% NaOCl soak as Humaydan, Harman, Nedrow and Di Nitto (1980) reported that treatment of brassica seeds with the weak oxidising agent reduced the phytotoxic effect of streptomycin sulphate on brassica seedlings.

Conclusions

Streptomycin sulphate was the only seed treatment used in this trial which completely controlled all *P.syringae* pv *tomato* in association with the seed.

Bashan *et al* (1978) reported that *P.syringae* pv *tomato* was present on seed as a seed contaminant because the 'vacuum surface sterilisation' technique used in their work inactivated all *P.syringae* pv *tomato*. This conclusion is questioned because the 'surface sterilisation' may also inactivate bacteria infecting the seed as a result of some of the germicide being vacuum infiltrated into the seed.

The surface sterilisation treatments used in this study, failed to completely inactivate all the bacteria in both artificially inoculated and commercial 'Moneymaker' seedlines, indicating that *P.syringae* pv *tomato* can survive on or in the seed, in positions where it is protected from the surface sterilant. It is likely that most infested tomato seeds are contaminated with the pathogen but this work suggests that *P.syringae* pv *tomato* can probably also infect a low percentage of seeds.

Acid extraction inactivated most of the *P.syringae* pv *tomato* in association with the seed and is a very useful seed extraction process. However, because the pathogen surviving in association with a small percentage of the seed could be responsible for an epidemic, acid extraction is inadequate as a seed treatment for control of *P.syringae* pv *tomato*. Sodium hypochlorite and Dichlone 50W^{R} have similar short comings.

Streptomycin sulphate seed treatments gave complete control of *P.syringae* pv *tomato* associated with the seed, but because prolonged exposure to streptomycin sulphate reduced seedling vigour they can not be applied in the seed extraction or cleaning process. However, the slurry seed treatment could easily be applied by nurserymen and growers to all seedlines sown, just prior to planting. The cost of streptomycin sulphate to treat the tomato seed (0.35c per kilogram of seed in 1980), is minimal compared to the savings which may be made if speck free seedlings are produced.

The use of streptomycin sulphate as a seed treatment is unlikely to create problems with resistance, provided the antibiotic is not used as a field treatment in speck infected areas. Regular exposure of *P.syringae* pv *tomato* to the antibiotic, or exposure to increasing concentrations of the antibiotic, could increase the possibility that *P.syringae* pv *tomato* will become resistant to the drug. Bacteria gain resistance to a drug by transferable plasmids (Bacon pers. comm. 1980) and the chances of this occurring are increased as more bacteria are exposed to the drug. If all seed was acid extracted prior to streptomycin sulphate treatments, then very few bacteria (only those surviving acid extraction) would be exposed to streptomycin sulphate so the possibility of resistant bacteria resulting from exposure to the drug is minimised.

As treating tomato seed with streptomycin sulphate is a relatively simple, low cost operation it is recommended that all tomato seedlines, regardless of their disease history, should be acid extracted to control TMV and some *P.syringae* pv *tomato*. The seed should also be treated

at 2.5 g (745 units/mg) of streptomycin sulphate per kilogram of seed, by the nurseryman or grower, to control any remaining *P.syringae* pv *tomato* and ideally sown immediately. Unfortunately this is not possible at present, because streptomycin sulphate is not registered by the New Zealand Agriculture Chemicals Board as a seed treatment for tomatoes. However, the author recommends that all tomato seed should be acid extracted and streptomycin sulphate treated (subject to registration for this purpose) at least until a satisfactory alternative is developed.

4. SOIL AND PLANT DEBRIS

Introduction

A number of researchers (Bryan 1933, Grogan *et al* 1974, Chambers and Merriman 1975, Bosshard-Heer and Vogelsanger 1977, Schneider and Grogan 1977a, Bashan *et al* 1978) have reported that *P.syringae* pv *tomato* can survive in and be isolated from soil and plant debris. Grogan *et al* (1974) regularly isolated *P.syringae* pv *tomato* from field soils where crops with a high incidence of bacterial speck were grown. The pathogen survived in buried infected plant debris throughout the winter and speck symptoms regularly developed on seedlings grown in infested soils. Grogan *et al* (1974) considered that the soil is probably the main source of inoculum for the next seasons crop. Chambers and Merriman (1975) reported that the main carry-over between crops appeared to be in the soil and in plant debris. Grogan *et al* (1974) and Schneider and Grogan (1977a) reported that *P.syringae* pv *tomato* can infect tomato roots and concluded that the pathogen may be able to survive with root debris in the soil. Schneider and Grogan (1977a) also reported that the pathogen can be ubiquitous because it was isolated from soils with no known history of tomato culture. Bosshard-Heer and Vogelsanger (1977) noticed that the pathogen survived better in sterilised soil than in non-sterilised soil samples. In addition to soil properties such as pH and nutrient content, the incubation temperature was the most influential factor determining the longevity of the pathogen in the soil; soil moisture was relatively unimportant (Bosshard-Heer and Vogelsanger 1977). Bashan *et al* (1978) regularly isolated *P.syringae* pv *tomato* from soils where diseased crops were grown in the previous season.

The work of these researchers indicate that the soil and plant debris are important sources of inoculum in some parts of the world. The importance of the soil and plant debris as sources of inoculum in New Zealand has not been determined. Accordingly this research aimed to determine whether (i) the pathogen can survive in association

with the soil and (ii) if the soil provides a source of primary inoculum in the new seasons crop in New Zealand.

Materials and Methods

A. Direct isolation

Trial 1

A 50:50 peat-sand planting mix, containing a full compliment of nutrients, was inoculated with a turbid 24h suspension of *P. syringae* pv *tomato* by mixing 15 ml of the virulent bacterial suspension with ca. 300g of planting mix. Unless otherwise stated all inoculated soils used in the soil work were inoculated in this manner. The soil mix was kept moist and incubated for a week at 20-25C.

After one weeks incubation isolations were made from the soil by diluting 1g of soil with 9 ml of 0.85% sterile saline and agitating on a magnetic stirrer for 10 mins. The four replica solutions were diluted 1 in 1,000, 2,000 and 5,000 and 1 ml of each dilution was plated to each of 5 Kings B medium and 5 D4 agar plates. In all isolation work from the soil the King's B medium and the D4 agar was made more selective or more differential by the addition of penicillin or crystal violet respectively. All D4 plates were incubated at 25C for 2-3 days. All colonies with similar morphology to *P. syringae* pv *tomato* on D4 agar were steaked to King's B medium agar. Blue fluorescent colonies on King's B medium agar were checked for oxidase production and oxidase negative colonies were pathogenicity tested on tomatoes. Unless otherwise stated all direct isolations from the soil were made in this manner.

Trial 2

One thousand 'VF 145-B-7879' seeds, vacuum infiltrated with a virulent isolate of *P. syringae* pv *tomato*, were mixed with ca. 300g of the peat-sand planting mix. The soil-seed mixture was moistened and incubated at 25C for a week and then the seed was separated from the soil with the aid of a 1.4mm mesh strainer. Isolations

were made from the soil mix.

Trial 3

Friable silt-loam samples were collected from 3 locations within a severely infected tomato crop on a Manawatu river terrace at Aokautere. In addition to testing ten 1g samples of the soil from each of the 3 locations, two 10g soil samples, from each location, were added to 90 ml of 0.85% sterile saline and tested.

Trial 4

A sample of dried sewerage, which is sold as a garden fertiliser in Nelson, was forwarded to the author for sampling. This fertiliser was suspected of being a source of primary inoculum of *P. syringae* pv *tomato* and could possibly be responsible for the increased appearance of bacterial speck in that area.

The dried sewerage sample was tested using three methods:

i) Ten grams were added to 90 ml of sterile saline and tested as in Trial 1.

ii) Four 10g sewerage samples were added to 90 ml of sterile water in four 150 ml Erlenmyer flasks and shaken overnight on an orbital shaker at 80 rpm. The resulting suspension was filtered, under vacuum, through Whatman No. 1 filter paper in a Buchner funnel and washed through with sterile water. The filtrate of each sample was collected and spun at 27,500 g for 15 mins. using a GSA rotor. One millilitre of the supernatant of each sample was enriched in 10 ml of D4 broth and the rest of the supernatant was discarded. The pellet of each sample was resuspended in 15 ml of sterile water and 1 ml was added to 10 ml of D4 broth in each of 5 McCartney bottles. All D4 broth cultures were enriched for 36h at 25C and then diluted 1:100 and 1:1000 to King's B medium agar. All blue fluorescent colonies were tested for oxidase production.

iii) Four 10g sewerage samples were tested in the manner described with the exception that the samples were added to 90 ml of King's B medium broth in Erlenmyer flasks.

B. Indirect isolation from soils (Baiting)

Trial 1

Treatment 1

The peat-sand planting mix was artificially inoculated. One hundred pathogen free 'VF 145-B-7879' seeds were sown to each of four 140 x 35 x 45 mm seedling trays and germinated in a mist chamber at 25C. The cotyledons and first leaves were gently injured with a cottonwool bud and returned to the mist chamber for a week. Isolations were made from the foliage and roots of the seedlings by macerating in sterile water and streaking to King's B medium or by incubating overnight on an orbital shaker in D4 broth and streaking to King's B medium agar. All King's B medium agar plates were incubated for 2 days at 25C and observed for fluorescence under ultra-violet light. Blue fluorescent isolates were tested for oxidase production with oxidase negative colonies being tested for pathogenicity to tomatoes. This procedure was used for all isolations and identifications made in the planting media baiting work 'VF 145-B-7879' seed was germinated in autoclaved soil in a mist chamber as a control.

Treatment 2

'Fireball' seed was sown to the surface of the peat-sand planting mix in four 140 x 85 x 45 mm seedling trays. The seed was covered with a thin layer of artificially inoculated planting mix. The seeds were germinated in a mist chamber at 25C and the cotyledons and first leaves were gently injured. Seedlings were sampled 1-2 weeks after injury. 'Fireball' seed was sown to and covered with autoclaved soil, germinated, injured and the seedlings were sampled as controls.

Treatment 3

The peat-sand planting mix was inoculated with *P. syringae* pv *tomato* and 100 three week old pathogen free 'UC 134' seedlings were transplanted into the soil mix in two 460 x 330 x 55 mm seedling trays.

Twenty seedlings were transplanted to autoclaved soil as a control. All seedlings were incubated on the glasshouse bench for the first week. After 1 week the roots of ten plants were sampled for *P.syringae* pv *tomato* and the remaining plants were incubated at 25C in the mist chamber for two weeks when the roots, stems and foliage of 6 inoculated seedlings and 4 control seedlings were sampled.

Trial 2

The 3 soil samples collected from the Manawatu river terrace, were combined 50:50 with the sterile peat-sand planting mix so there was enough soil to fill a 460 x 330 x 55 mm seedling tray. One hundred three week old pathogen free 'UC134' seedlings were transplanted into this mix and incubated in a mist chamber at 25C for 4 weeks. Usually only the roots of the 15 plants were sampled but the foliage of two plants was also tested. Seedlings were transplanted to an autoclaved soil sample as a control.

Results and Discussion

A. Direct isolations

Trial 1

Although the soil was inoculated with a turbid bacterial suspension, the pathogen was only isolated from one sample. If the pathogen had multiplied in the soil a higher recovery rate would be expected. Therefore, the pathogen probably only survived in the soil and even then the population declined considerably after inoculation. Bosshard-Heer and Vogelsanger (1977) reported that *P.syringae* pv *tomato* survived for longer than 26 days in most of the non-sterile soils inoculated and incubated at 20C, therefore it was expected that *P.syringae* pv *tomato* would survive for longer than a week in the peat-sand planting mix.

Trial 2

A number of blue fluorescent colonies were isolated from the soil but only one was oxidase negative. This colony was pathogenic to tomatoes producing symptoms typical of bacterial speck.

P.syringae pv *tomato* can infect seedlings as a result of its association with the soil (Grogan *et al* 1974) Therefore, because the pathogen can infest the soil when it is introduced with the seed it should be possible for seed-soil-plant transfer to occur. *P.syringae* pv *tomato* could be introduced into a new area or crop because of its association with the seed and thus, seedling infection could result from seed-soil-plant transfer.

Trial 3

A large number of blue fluorescent colonies were isolated to King's B medium agar but none of these were oxidase negative.

It was not possible to conclude that the soil was *P.syringae* pv *tomato* free, although the limited soil sample screened may not have been infested, the pathogen could have infested some of the soil the crop was growing in.

Trial 4

All isolation methods resulted in the appearance of a large number of blue fluorescent colonies on King's B medium agar. None of the blue fluorescent colonies were oxidase negative thus *P.syringae* pv *tomato* was not isolated.

Because *P.syringae* pv *tomato* was not isolated from the sewerage samples does not mean the pathogen was unable to survive in or be disseminated in the sewerage. However, the author doubts that any *P.syringae* pv *tomato* entering the sewerage treatment station, in association with the sewerage, would survive the anaerobic fermentation

and heat treatments used to refine sewerage to a dried garden fertiliser. The increased incidence of bacterial speck in the Nelson area may be due to the use of infested seed and the appearance of the disease in gardens, using the sewerage as a garden fertiliser may be coincidental.

The pathogen was isolated from artificially inoculated soils a short time after inoculation but was not isolated from any soils suspected of naturally harboring the pathogen. Thus, all this work indicates is that *P. syringae* pv *tomato* can survive in association with a peat-sand planting mix for short periods of time under glasshouse conditions in New Zealand.

B. Indirect isolation from soils (Baiting)

Trial 1

Treatments 1 and 2

No bacterial speck symptoms were apparent on any of the seedlings germinated in Treatments 1 or 2. Blue fluorescent colonies were isolated to King's B medium agar from the seedlings in Treatment 1. Four colonies were oxidase negative but none were pathogenic to tomatoes. Blue fluorescent oxidase positive colonies were isolated from the control seedlings in Treatment 1. No blue fluorescent colonies were isolated to King's B medium agar from either control or seedlings in inoculated soil in Treatment 2.

The pathogen may not have been isolated because it did not contact the foliar plant parts. Gentle injury of the seedlings foliage did not appear to favour infection as expected. The pathogen was not isolated from the roots possibly because they were not injured, a process which might favour infection.

Treatment 3

No symptoms resembling those of bacterial speck were apparent on any of the seedlings prior to incubation in the mist chamber. However, blue fluorescent colonies were isolated from the washed roots of two plants. One colony was oxidase negative and was pathogenic to tomatoes producing symptoms typical of bacterial speck in pathogenicity tests. After incubation in the mist chamber no lesions developed upon the leaves but some lesions appeared at ground level, on the stems, of some seedlings. These lesions were similar to the type of lesions produced in post-emergence damping off with the collar like necrotic area eventually causing seedling collapse. A number of blue fluorescent oxidase negative colonies were isolated from either root or stem samples from seedlings with stem lesions (Table 26). Attempts to induce this collar-rot like symptom by inoculating the stems of 'UC134' seedlings with *P. syringae* pv *tomato* were unsuccessful. No symptoms appeared in any of the control seedlings and no blue fluorescent, oxidase negative colonies were isolated from the 4 seedlings tested.

Table 26: Isolation of *P. syringae* pv *tomato* from transplanted tomato seedlings

Plant part sampled	Health of plant sampled	No. of seedlings sampled	No. of Blue fluorescent isolates	Oxidase reaction	Pathogenicity
leaf	lesioned stem	4	0		
stem	lesioned stem	4	24	11-	11+
root	lesioned stem	4	22	10-	10+
leaf	healthy	2	0		
stem	healthy	2	0		
root	healthy	2	2	2+	
leaf	control	4	0		
stem	control	4	0		
root	control	4	2	2+	

P.syringae pv *tomato* was isolated from the roots and stems of the transplanted seedlings possibly because both roots and stems were injured, during transplanting, and this favoured infection. In New Zealand many tomato plants are produced from transplants so the pathogen may infect the roots, stems and possibly the foliage during transplanting. This work demonstrates that soil-plant transfer, to the roots and stems of transplanted seedlings, can occur from artificially inoculated soil.

Trial 2

No lesions appeared on any seedling parts but blue fluorescent colonies were isolated from most seedlings sampled. Only two blue fluorescent isolates were oxidase negative, both producing speck like symptoms when pathogenicity tested on tomatoes. Both positive isolations were made from the roots. No blue fluorescent colonies were isolated from the control seedlings.

The pathogen was not isolated from the soil sample by the direct isolation method but was isolated from the sample when the transplanted seedlings were used as bait. This work demonstrates that the soil can act as a source of inoculum for *P.syringae* pv *tomato* in New Zealand.

The survival of *P.syringae* pv *tomato* in naturally infested soil was not tested. However, presumably under favourable conditions survival could be similar to that reported in other countries of the world. Most areas where the survival of *P.syringae* pv *tomato* in the soil has been reported have arid climates so the survival of the pathogen in the wetter climate of New Zealand may be affected.

Conclusion

If a grower plants bacterial speck free transplants into a soil infested with *P.syringae* pv *tomato* then it is possible that seedling infection will occur. Soil-plant transfer may prevent growers using

bacterial speck free transplants, from growing disease free plants in New Zealand. Although infection may occur, with the soil as the primary source of inoculum, it does not decrease the necessity to use pathogen-free seed as the production of healthy transplants delays the onset of disease, reduces the yield losses which result from early infections (Grogan *et al* 1974) and reduces the loses which can occur in the nursery.

In New Zealand it is not economical to sterilise field soils to control *P. syringae* pv *tomato*. However, in the nursery situation and in glasshouse plantings soil sterilisation could enable the production of disease free seedlings or plants. No work has been done on the effect of soil sterilant treatments on the survival of *P. syringae* pv *tomato*. Although *P. syringae* pv *tomato* can survive in the soil in New Zealand for short periods of time, its ability to survive prolonged incubation in the soil and the importance of the soil as a primary source of inoculum has not been established.

In this study no work was done on the survival of *P. syringae* pv *tomato* in plant debris but it is likely that the pathogen, in common with some other foliar bacterial pathogens, can survive in infected plant debris, thus providing a source of inoculum in New Zealand. However, because decomposition of plant debris is likely to be much more rapid in the wetter climate of New Zealand than in some of the countries with arid climates where research on this aspect of the disease has been undertaken, the pathogen is not likely to survive in association with plant debris in this country for as long as it does in a dry climate.

5. ALTERNATIVE HOSTS

Introduction

P.syringae pv *tomato* can survive as resident populations on tomato leaves and is also reported to be ubiquitous (Schneider and Grogan, 1977a). As the pathogen is ubiquitous it should be able to survive as resident populations or as epiphytes on alternative hosts and possibly infect some species. Bryan (1933) reported that inoculations of seedlings and well grown leaves and fruit of eggplant, bell peppers, pimentos, bean, cucumber and squash failed to produce any infections. Unfortunately isolations from the unlesioned leaves were not attempted so the ability of the pathogen to survive on these plants was not reported. *P.syringae* pv *tomato* causes a hypersensitive reaction when inoculated to *Nicotiana tabacum L.* (tobacco) and this reaction is used in some determinative schemes for identification (Lelliott *et al* 1966).

Grogan *et al* reported that plants other than tomato may be involved in the soil population dynamics of *P.syringae* pv *tomato*. Schneider and Grogan (1977a) isolated *P.syringae* pv *tomato* from the leaves of *Brassica campestris* L. (wild turnip), *Brassica nigra* (L.) Koch (black mustard) and *Erodium cicutarium* (L.) L'Her (redstem filaree) all growing in a 40 year old prune orchard. It was also isolated from the roots of *Triticum vulgare* L. (wheat), *Beta vulgaris* L. (sugar beet), *Matricaria matricariodes* (Less.) Parker (pineapple weed), *Erodium botrys* (Cav.) Bertol. (broad leaf filaree), redstem filaree and tomato, both in areas with and without a previous history of tomato culture. None of these plants showed any symptoms of bacterial speck.

P.syringae pv *tomato* is not the only pseudomonad which is able to survive as an epiphyte on other hosts. This ability has been reported for other pathovars of the *P.syringae* species (Leben 1974; Ercolani, Hagedorn, Kellman and Rand 1974; Schneider and Grogan 1977a; Latorre and Jones 1979 a & b).

In this study attempts were made to inoculate and isolate the pathogen from a number solanaceous plants and from a number of weed species collected from within a bacterial speck infected crop.

Materials and Methods

A. Isolation from inoculated plants

Eight plants of each species, namely *Capsicum frutescens* L. (green pepper), *Cucumus sativus* L. (cucumber), *Datura stramonium* L. (thornapple), *Nicotiana glutinosa* L., *N. tabacum* L. (tobacco), *Petunia hybrida* Vilm., *Solanum melangena* L. (egg plant), *Solanum muricartum* L. (pepino), *Solanum nigrum* L. (black nightshade), and *Solanum tuberosum* L. (potato), were inoculated with a turbid 24h virulent culture of *P.syringae* pv *tomato*. One plant of each species was inoculated with sterile water as a control. All expanded leaves and cotyledons were inoculated by gently rubbing with a cottonwool bud. Four plants of each species were incubated on a glasshouse bench at 20-25C and overhead watered daily. The other four plants of each species were incubated in a mist chamber at 20-25C. All plants were regularly observed for symptom production and two plants from the glasshouse bench and two plants from the mist chamber were sampled 2 and 4 weeks after inoculation. Plants were sampled either by macerating inoculated and non-inoculated leaves in sterile water and streaking to King's B Medium or by enriching inoculated and non-inoculated leaves in D4 broth on an orbital shaker at 80 rpm over night and streaking to King's B medium agar. All blue fluorescent colonies were tested for oxidase production and all oxidase negative colonies were pathogenicity tested on tomatoes.

B. Isolation from symptomless plants

A number of plant species, namely *Amaranthus powellii* L. (red root) wild turnip, *Capsella bursa-pastoris* (L.) Med. (shepherds purse), *Chenopodium album* L. (fathen), *Coronopus didymus* L. (twincress), *Datura stramonium* L. (thornapple), *Malva sylvestris* L. (mallow),

Nicandra physilodes L. (apple of peru), *Polygonum persicaria* L. (willow weed), *Rumex crispus* L. (dock), *Senecio vulgaris* L. (groundsel), black nightshade, potato and *Taraxacum officinale* Weber (dandelion) were collected from within a severely speck infected crop at Aokautere on a Manawatu river terrace. The roots and foliage of 4 plants of each species were sampled. All soil adhering to the plants was washed off with sterile water and plant parts were sampled in the manner used to isolate from inoculated plants.

Results and Discussion

A. Isolation from inoculated plants

P.syringae pv *tomato* was re-isolated from most plant species sampled both 2 and 4 weeks after inoculation (Table 27) and was never isolated from the control plant or non-inoculated leaves.

The pathogen induced a hypersensitive reaction in both *N. glutinosa* (Plate 14) and tobacco. Symptoms typical of bacterial speck of tomatoes developed on young egg plant leaves (Plate 15) but lesions typical of those caused by *P.syringae* pv *tomato* were not produced on any other plants. However, both green pepper and thornapple showed hormone-like symptoms after inoculation. Both the water inoculated control and *P.syringae* pv *tomato* inoculated peppers initially showed a down curling of the central leaf vein followed by a downward curling of the leaf lamina. All new leaves formed after inoculation developed this symptom. Non-inoculated plants showed no symptoms. A shoestring effect and distortion occurred in the thornapple plants inoculated with *P.syringae* pv *tomato* and this symptom was apparent in all new leaves. The cause of this condition was not identified.

B. Isolation from symptomless plants

No blue fluorescent, oxidase negative colonies were isolated from any plant parts sampled from any of the weed species collected from within a bacterial speck infected tomato crop.

This research has demonstrated that *P.syringae* pv *tomato* can survive on alternative hosts as an epiphyte and, although Bryan (1933) reported no symptoms were apparent in eggplant, it can infect and produce lesions typical of bacterial speck lesions on the young inoculated leaves of eggplant in New Zealand. Because the pathogen was not isolated from any of the plant species sampled from a diseased crop does not mean it cannot naturally survive in association with other plant species. This work has demonstrated that *P.syringae* pv *tomato* can survive on a number of the plant species tested for at least four weeks under the artificial conditions of the experiment. The pathogen was not isolated from any plants collected from diseased fields so the importance of alternative hosts as sources of primary inoculum in New Zealand was not established. Although no explanation can be given for these results they are rather surprising if the findings of Schneider and Grogan (1977a) are considered.

Plate 14: Hypersensitive reaction of *N. glutinosa* (right) to infestation by *P. syringae* pv *tomato*. Healthy control leaf (left).

Plate 15: Bacterial speck symptoms on artificially inoculated egg plant leaf (right) and a healthy leaf (left).

Table 27: Isolation of *P.syringae* pv *tomato* from inoculated leaves of some plant species

Plant species	Incubation conditions	Time after inoculation (weeks)	No. of blue fluorescent isolates	Oxidase reaction	Pathogenicity
C.frutescens	G[1]	2	3	3-	3+
	M[2]	2	0		
	G	4	0		
	M	4	4	4-	4+
C.sativus	G	2	0		
	M	2	2	2-	2-
	G	4	0		
	M	4	0		
D.stramonium	G	2	2	2-	2+
	M	2	1	-	+
	G	4	0		
	M	4	3	3-	3+
N. glutinosa	G	2	20	20-	20+
	M	2	9	9-	9+
	G	4	5	5-	5+
	M	4	15	15-	15+
N.tabacum	G	2	whole plate	-	+
	M	2	" "	-	+
	G	4	2	2-	2+
	M	4	0		
P.hybrida	G	2	1	+	
	M	2	4	4-	4+
	G	4	4	4-	4+
	M	4	2	2+	
S.melangena	G	2	whole plate	-	+
	M	2	" "	-	+
	G	4	" "	-	+
	M	4	" "	-	+
S.muricartum	G	2	4	4-	4+
	M	2	2	2-	2+
	G	4	5	5-	5+
	M	4	8	8-	8+
S.nigrum	G	2	4	4-	4+
	M	2	0		
	G	4	0		
	M	4	1	+	
S.tuberosum	G	2	0		
	M	2	0		
	M	4	1	+	

[1] G = incubation on a glasshouse bench

[2] M = incubation in a mist chamber

6. SYSTEMIC NATURE OF *P.SYRINGAE* PV *TOMATO*

Introduction

Some research has been done to determine if *P.syringae* pv *tomato* can infect tomatoes systemically. Grogan *et al* (1974) isolated the pathogen from necrotic root lesions and reported that it did not invade the plant systemically. Bashan *et al* (1978) were unable to demonstrate infection of the vascular system by isolating from leaves and vascular tissues and concluded that *P.syringae* pv *tomato* was a local lesion pathogen. Thus bacterial speck is similar to most other bacteria causing leaf spot diseases which apparently do not move through the plant vascular system (Goodman, Kiraly and Zaitlin 1967).

Some bacterial diseases caused by *P.syringae* pathovars are reported to be systemic, including *Pseudomonas syringae* pv *lachrymans* causing angular leaf spot of cucumber (Pohronezny *et al* 1977) and a tumour inducing bacteria *Pseudomonas syringae* pv *savastanoi* (Wilson and Magie, 1964). The author of this thesis could see no reason why *P.syringae* pv *tomato* could not invade tomato plants systemically, especially when other pathovars of *P.syringae* can infect their hosts systemically. Consequently the systemic invasion of host plants by *P.syringae* pv *tomato* was investigated.

Materials and Methods

Five week old 'UC 134' tomato seedlings were inoculated with *P.syringae* pv *tomato* in each of the methods described.

(i) The tomato leaves of 8 plants were inoculated by injecting a turbid 24h culture of *P.syringae* pv *tomato* into the midvein, with a fine gauge hypodermic needle, until a small visibly water soaked area appeared around each inoculation site (Miller 1975).
The inoculum was allowed to move slowly out of the syringe into the plants vascular system. The inoculated leaves of four plants were enclosed in plastic bags and the inoculated midvein areas on the

other four plant's leaves were covered with petroleum jelly. (Pohronezny *et al* 1977). Two plants were inoculated with sterile water and the leaves of one plant were enclosed in a plastic bag; the inoculation sites on the other plant were covered with petroleum jelly as controls.

(ii) The stems of eight tomato seedlings were inoculated with the turbid 24h bacterial suspension by injecting into the vascular system in a number of positions between the first and second leaf internodes. The stems of four plants were wrapped in parafilm and the inoculation sites on the other four plants were covered with petroleum jelly. Two plants were inoculated with sterile water and wrapped in parafilm or covered with petroleum jelly as controls.

(iii) The stems of eight tomato seedlings were injected with the turbid 24h bacterial suspension just above ground level. The inoculation sites of four plants were wrapped with parafilm and on the other four plants were covered with petroleum jelly. Two plants were inoculated with sterile water as controls and the inoculation sites were covered with parafilm or petroleum jelly.

(iv) The roots of eight tomato seedlings were inoculated by removing the seedling from the pot and carefully injecting the bacterial suspension into the root system. Two plants were inoculated with sterile water as controls. All seedlings were repotted and aluminium foil was wrapped tightly around the stem and the top of the pot (Plate 16).

Because eggplant was found to be susceptible to *P.syringae* pv *tomato* five week old eggplants were inoculated by injecting a turbid suspension of the pathogen into the mid leaf vein of three eggplants and into the stems of three plants just above ground level. Leaves and stems of two plants were inoculated with sterile water as controls. All leaf inoculation sites were enclosed in plastic bags and all stem inoculation sites were wrapped in parafilm.

Plate 16: Tomato plant showing the tinfoil wrapped around the pot and the lower plant stem after root inoculation

All inoculated plants were incubated on a glasshouse bench at 20-25C for 2 weeks and capillary watered to prevent any bacterial spread by water splash. The plants were removed from the pots and all adhering soil was washed off the roots. All plants were sampled by taking non-inoculated leaf samples, stem samples from just below the apical meristem, stem samples from just above ground level or 1 cm above the low stem inoculation site, and non-inoculated root samples.

Samples were surface sterilised by emersion in 1% sodium hypochlorite for 5 minutes and rinsed in sterile water for 1-2 mins. The epidermal cells of all stem samples were scraped off with a sterile scalpel blade. All samples were macerated in sterile water and streaked to each of 2 King's B medium agar plates. Blue fluorescent colonies were tested for oxidase production with oxidase negative colonies being tested for pathogenicity to tomatoes.

Results and Discussion

P.syringae pv *tomato* was isolated from only one plant part sampled on the tomato plants, a high stem sample from about 1 cm above the inoculation site (Table 28). No blue fluorescent colonies were isolated from the control plants.

Because *P.syringae* pv *tomato* was not regularly isolated from the tomato plant parts sampled this work supports the views of other researchers that the pathogen does not become systemic.

If *P.syringae* pv *tomato* were systemic in tomatoes this could explain how the pathogen becomes associated with the seed. The speck lesions on the fruit do not penetrate deeply (Bryan 1933), and as this current work indicates the pathogen is probably not systemic it is not known how the pathogen becomes associated with the seed. The only other way the pathogen may contact the seed is during the extraction process.

Table 28: Isolation of *P. syringae* pv *tomato* from leaves, stems and roots of inoculated tomato plants.

Plant part inoculated	Plant part isolated from	No. of plants	No. of blue fluorescent isolates	Oxi-dase reaction	Pathogeni-city
leaf	R[1]	8	0		
leaf	Lo[1]	8	2	2+	
leaf	Hi[1]	8	0		
leaf	Le[1]	8	0		
high stem	R	8	2	2+	
high stem	Lo	8	0		
high stem	Hi	8	1	-	+
high stem	Le	8	4	4+	
low stem	R	8	2	2+	
low stem	Lo	8	0		
low stem	Hi	8	0		
low stem	Le	8	4	4+	
root	R	8	1	+	
root	Lo	8	6	6+	
root	Hi	8	0		
root	Le	8	0		

[1] R = root Lo = low stem Hi = high stem Le= leaf

The pathogen was isolated from eggplant parts other than those it was inoculated to, even when the inoculation sites were wrapped in parafilm (Table 29). It is possible that the pathogen could have been spread by water splash to other plant parts when washing or sampling the plants. No blue fluorescent colonies were isolated from any of the eggplant controls.

Table 29: Isolation of *P.syringae* pv *tomato* from roots, leaves and stems of inoculated eggplants

Plant part inoculated	Plant part isolated from	No. of plants sampled	No. of blue fluorescent isolates	Oxidase reaction	Pathogenicity
leaf	R	3	0		
leaf	Lo	3	4	3-	2+
leaf	Hi	3	1	-	+
leaf	Le	3	0		
low stem	R	3	0		
low stem	Lo	3	2	2-	2+
low stem	Hi	3	1	-	+
low stem	Le	3	0		

This work indicates that *P.syringae* pv *tomato* is probably not systemic but it does not provide conclusive evidence that the pathogen is not systemic in tomatoes or other plant species. Further studies may show that the pathogen can be systemic and this could explain how the pathogen becomes associated with the seed.

APPENDIX I

Composition and Preparation of Culture Media and Reagents

All the media used were prepared as described and, in all cases, were sterilised by autoclaving at 121C, 15 psi for 15 minutes.

i. King's B Medium broth and agar (King *et al* 1954)

Glycerol	10g
Proteose peptone No . 3(Difco)	20g
Magnesium sulphate ($MgSO_4 7H_2O$)	1.5g
Dipotassium Hydrogen Orthophosphate (K_2HPO_4)	1.5g
Distilled water	1000 ml

Fifteen grams of agar (Davis) were also added when making solid media. The ingredients are dissolved and the pH is adjusted to 7.2. The broth medium is dispensed to McCartney bottles and autoclaved. The solid medium is autoclaved in 1 l flasks and cooled to 45C and poured or 20,000 units of penicillen per litre may be added before pouring to increase the selectivity of the agar.

ii. D4 broth and agar (Kado and Heskitt 1970)

Glycerol	10g
Casein hydrolysate	1g
Ammonium chloride (NH_4Cl)	5g
Disodium hydrogen orthophosphate (Na2 HPO4)	2.3g
Sodium dodecyl sulphate (reagant grade)	0.6g
Distilled water	1000 ml

Fifteen grams of agar (Davis) are added when making solid media. The addition of lppm crystal violet increased the differential capacity of the soild media. The selectivity of the broth can be increased by adding 2.5g of LiCl. The pH of the media is adjusted to 6.8 and the broth is dispensed to McCartney bottles and the agar to 1 l flasks and autoclaved. The solid media was cooled to 45C and poured.

iii. Sucrose agar (Lelliott *et al* 1966)

Nutrient agar	15g
Sucrose	5%
Distilled water	1000 ml

The components are mixed, autoclaved, cooled to 45C and poured.

iv. Water agar (Spiers pers.comm 1980)

Agar (Davis)	20g
Water	1000 ml

The medium was autoclaved cooled and approximately 15ml was added to each plate to give a thick solid medium.

v. Phosphate Buffered Saline Serology Agar (PBSSA)

Agar (Davis)	4g
Phosphate buffered saline	50 ml
Distilled water	950 ml

The medium was autoclaved, cooled to 45C and 0.5ml of 2% sodium azide was added before pouring the plates. Sodium azide prevents micro-organism growth on the medium.

vi. Tetrazolium Medium (Tuite 1969)

Agar (Davis)	17g
Peptone	10g
Casein hydrolysate	1.0g
Glucose	0.5g
Distilled water	900 ml

The medium is autoclaved, cooled to 45C and 100ml of an autoclaved stock solution of 2,3,5 triphenyl tetrazolium chloride is added then the plates are poured.

vii Oxidase reagant (Lelliott *et al* 1966)

Tetra methyl phenylene diamine hydrochloride	0.02g
Distilled water	2 ml

The solution is hygroscopic so is only made up in small amounts and is stored in small sealed bottles at 4C when not in use.

APPENDIX II

Origin of the seedlines used and the texture of the seedcoats

Ocean View Seeds Ltd,
263-265 St Aubyn St,
NEW PLYMOUTH.

'Beefsteak', smooth, fungicide treated.

Persson's Nurseries and Garden Centre,
500 Church St,
PALMERSTON NORTH.

'Moneymaker', hairy

Wattie Industries Ltd,
P.O. Box 439,
HASTINGS.

'Castlong' slightly hairy.
'Dorchester', smooth
'Scoresby', hairy, Spergon[R] treated.
'UC 134', slightly hairy
'VF 145-B-7879', smooth.

Webling and Stewart Ltd,
P.O. Box 38-570,
PETONE.

'Potentate', hairy, Selected DSIR stock
'Scoresby', hairy.

Arthur Yates & Co. Ltd,
270 Neilson St,
Onehunga,
AUCKLAND.

'Fireball', slightly hairy, slightly rough.

Seedlines hand and acid extracted in this study:

'Beefsteak', rough and hairy
'Castlong', rough and hairy
'Moneymaker', rough and hairy
'Scoresby', rough and hairy

APPENDIX III

Origins of Bacterial Isolates

Pseudomonas syringae pv *tomato*

C1 = Isolated from the cotyledon of a 'Beefsteak' seedling germinated from hand extracted seed, ex infected fruit.

C2 = Isolated from lesions on green 'Scoresby' fruit. March 1978

C3 = Isolated from lesions on green 'Scoresby' fruit. March 1978

C4 = Isolated from lesions on ripe 'Scoresby' fruit. March 1978

C5 = Isolated from 'Scoresby' leaf lesions. March 1978. Virulent culture

C6 = Isolated from 'Scoresby' leaf lesions. March 1978

C7 = Isolated from 'Beefsteak' leaf lesions. April 1978

C8 = Isolated from 'Beefsteak' fruit lesions. March 1978

C9 = Isolated from 'Moneymaker' leaf lesions. March 1978

C10 = Isolated from 'Moneymaker' fruit lesions. March 1978

Pseudomonas cichorii

1 = Culture 5707)Plant Diseases Division, Auckland

2 = Culture 5707) New Zealand.

Pseudomonas solanacearum

1 = Isolated from nutrient solution used in the hydroponic system in a glasshouse in Whangarei. July 1978

Pseudomonas species

I1 = Isolated from the soil. Blue fluorescent. Oxidase positive.

I2 = Isolated from the soil. Green fluorescent. Oxidase positive

I3 = Isolated from the soil. Green fluorescent. Oxidase negative

APPENDIX IV

Table 30: Detection of *P. syringae* pv *tomato* from tomato seeds, using an enrichment assay, Trial 2

Sample 1	No. of replicas	Fluorescence*	Oxidase Reaction	Pathogenicity test
500 autoclaved seeds	2	2 No growth		
2 seeds/500	3	3 Blue	3-	3+
2 seeds/500 ground	2	2 Green		
2 seeds/1000	3	2 Green 1 No growth		
2 seeds/1000 ground	2	1 Green 1 No growth		
2 seeds/2000	3	1 Blue 2 Green	-	+
2 seeds/2000 ground	2	2 Green		
2 seeds/4000	3	3 Green		
2 seeds/4000 ground	2	2 Green		
2 seeds/8000	3	2 Green 1 No growth		
2 seeds/8000 ground	2	2 Green		

* Fluorescence was either green or blue. Plates were recorded as blue fluorescent even if they only had one blue fluorescent colony. No growth was apparent on some plates.

Table 31: Isolation of *P.syringae* pv *tomato* from seed using the enrichment assay, Trial 3

Sample	Replicas	Fluorescence	Oxidase Reaction	Pathogenicity
50 bacteria/16 mls	2	Both green		
50 bacteria/8 mls	2	1 Blue, 1 Green	-	+
50 bacteria/4 mls	2	1 Blue, 1 Green	-	+
50 bacteria/2 mls	2	1 Blue, 1 Green	-	+
500 autoclaved ground seed	2	1 Green, 1 No growth		
10/500 seeds	2	2 Green		
10/500 seeds ground	10	3 Blue, 4 Green, 3 No growth	All +	
10/1000 seeds	2	1 Blue, 1 Green	+	
10/1000 seeds ground	10	3 Blue, 7 Green	All +	
10/2000 seeds	2	2 No growth		
10/2000 seeds ground	10	3 Blue, 6 Green, 1 No growth	All +	
10/4000 seeds	2	1 Green, 1 No growth		
10/4000 seeds ground	10	7 Blue, 2 Green, 1 No growth	All +	
supernatant 10/500 ground	4	2 Blue, 2 Green	++	
supernatant 10/1000 ground	4	1 Blue, 3 Green	+	
supernatant 10/2000 ground	4	3 Blue, 1 Green	-++	+
supernatant 10/4000 ground	4	2 Blue, 2 Green	++	

Table 32: Isolation of *P.syringae* pv *tomato* from seed using an enrichment assay, Trial 4

Sample	Replicas	No. of colonies sub-cultured	Fluor-escence	Oxidase Reaction	Patho-genicity
500 seeds (control)	2	0 (no growth)			
400 bacteria/14 mls	2	24 colonies	15 Blue	15-	15+
4 bacteria/14 mls	2	12 colonies	9 Blue	9-	9+
8 seeds/800 seeds	2	0			
8/1000	2	0			
8/2000	2	0			
8/4000	2	0			
8/8000	2	0			
4/8000	2	0			
2/8000	2	0			
1/8000	2	0			

Table 33: Effect of 2,4-D on tomato leaf discs and on the germination of 'Fireball' tomato seed

Treatment (% 24-D)	No. of seeds tested	% Germination and rate of germination	Effect on the leaf
H_2O	60	50 moderate	No obvious effect
0.025%	30	3 slow	No obvious effect
0.05%	30	3 slow	No obvious effect
0.1%	70	0	2 leaf discs brown and necrotic

FIGURE 2: MAXIMUM AND MINIMUM DAILY TEMPERATURES RECORDED AT THE DSIR, PALMERSTON NORTH

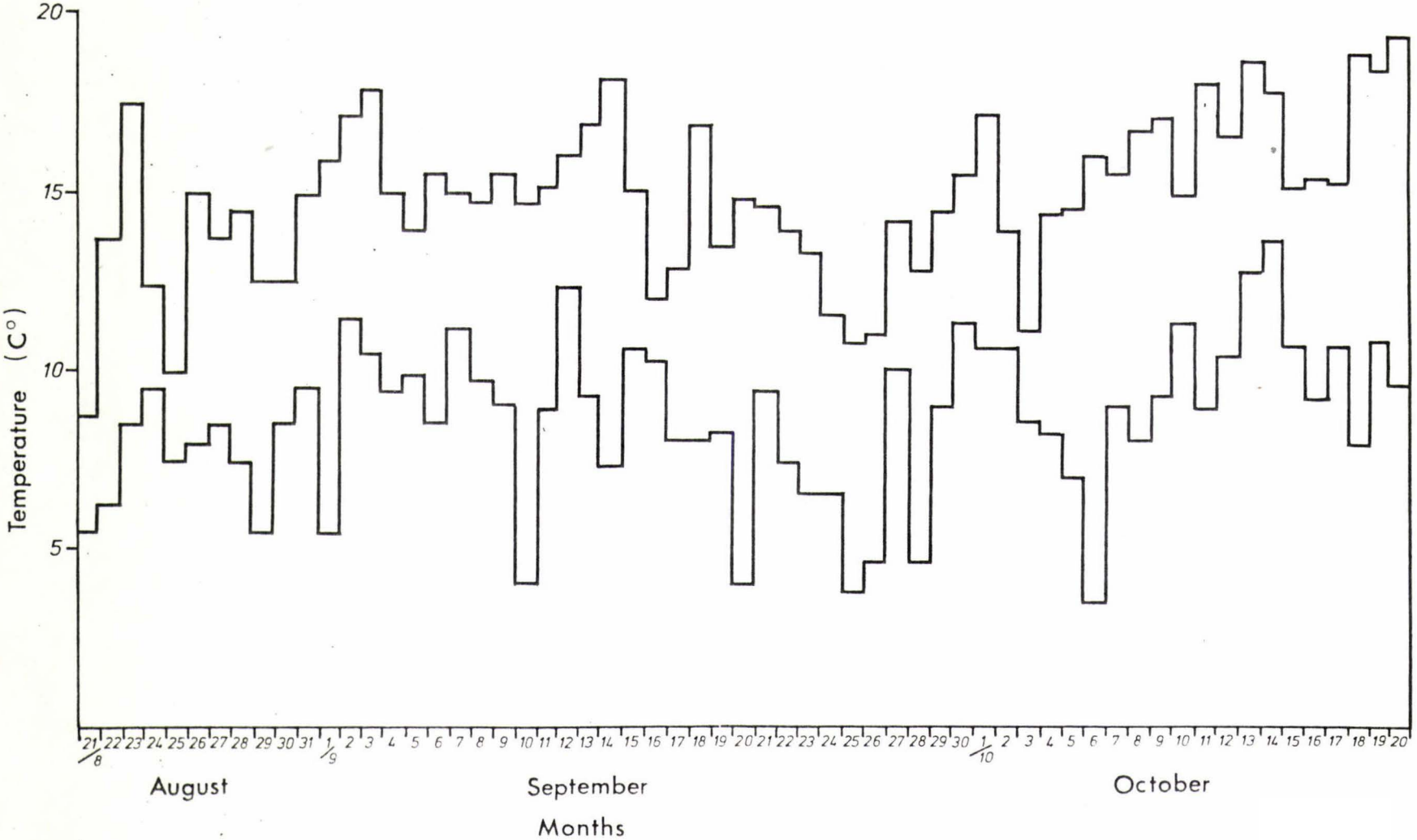


FIGURE 3: MEAN DAILY RELATIVE HUMIDITY RECORDED AT OHAKEA AIR FORCE BASE
DAILY RAINFALL 11.9.79 - 20.10.79 RECORDED AT DSIR, PALMERSTON NORTH

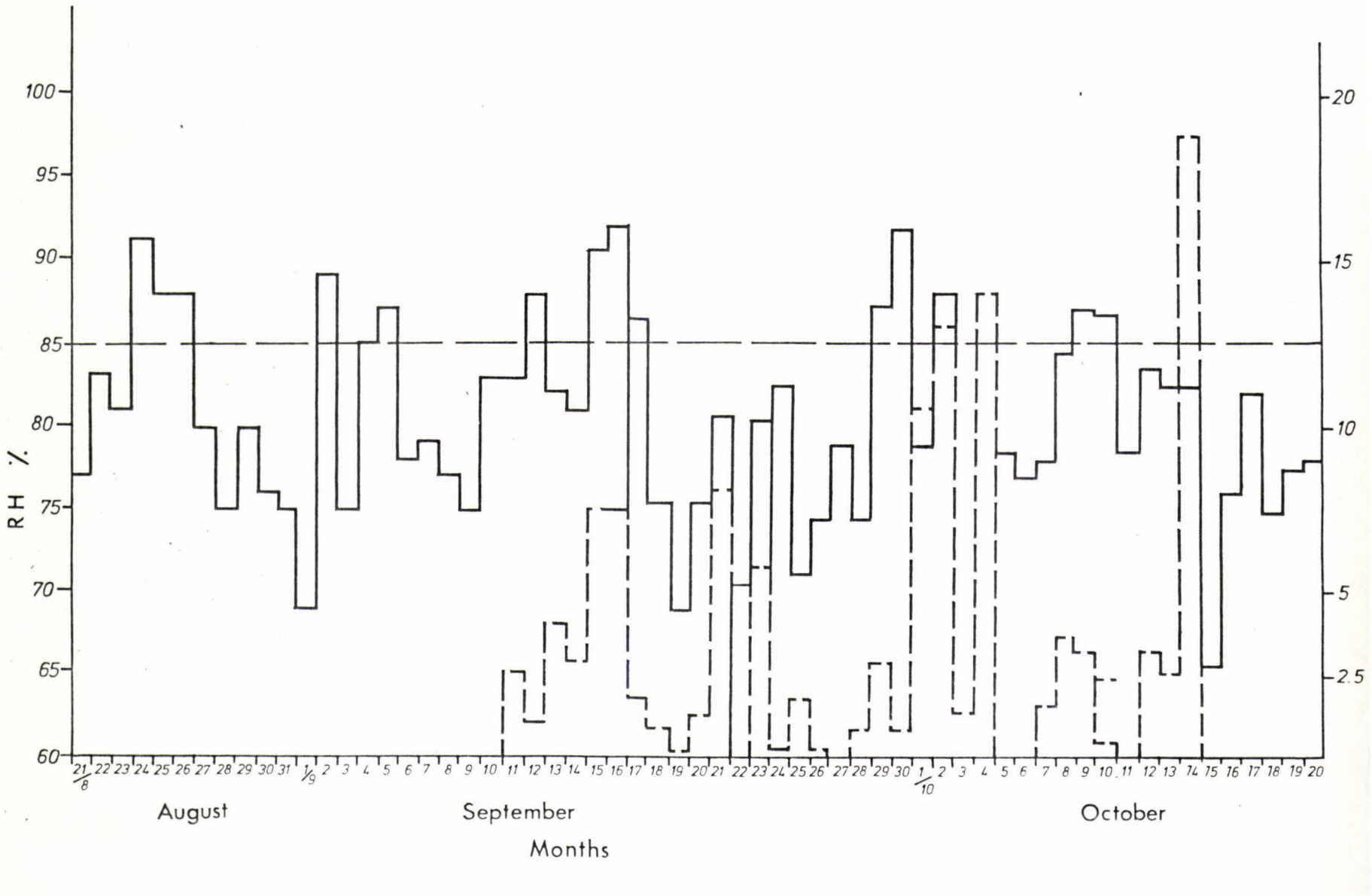
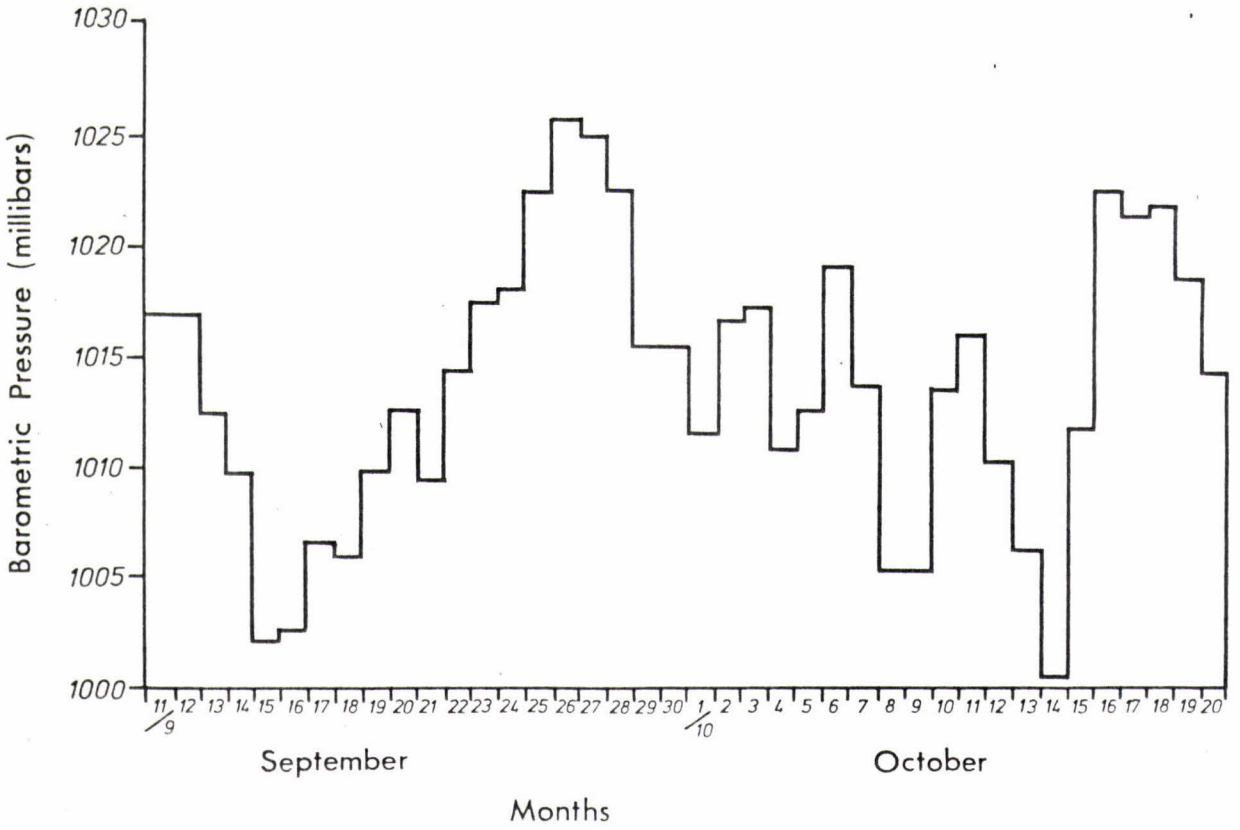


FIGURE 4: BAROMETRIC PRESSURE RECORDED AT OHAKEA AIR FORCE BASE 11.9.79 - 20.10.79



APPENDIX V

Other bacterial diseases of tomatoes observed

During this study on bacterial speck of tomatoes, two other bacterial diseases, causing significant losses in glasshouse tomato crops, were investigated.

One disease was present in a glasshouse crop in the Manawatu in the autumn of 1978. The symptoms on the tomato plants were similar to those reported by Wilkie and Dye (1974a) and both fruit and whole plant losses resulted from the infection. A number of green fluorescent bacteria were isolated and oxidase positive colonies were tested for pathogenicity to tomatoes. None of these colonies were pathogenic to tomatoes but because the symptoms so closely resembled those reported by Wilkie and Dye (1974a) it was presumed that the disease was stem bacteriosis caused by *Pseudomonas cichorii*. However, since this disease outbreak, a new disease, tomato pith necrosis, has been recorded in the United Kingdom (Scarlett, Fletcher, Roberts and Lelliott 1978). The disease symptoms, caused by the non-fluorescent pseudomonad *Pseudomonas corrugata* Roberts and Scarlett, are very similar to those produced by *P. cichorii* infection (Scarlett *et al* 1978). The disease could have been caused by the non-fluorescent pseudomonad, thus explaining why none of the fluorescent pseudomonads isolated were pathogenic to tomatoes.

The other disease caused a severe wilt in tomatoes grown in a hydroponic system under glass at Whangarei. Some diseased plant samples and a sample of the nutrient solution were forwarded to the author for testing. The signs and symptoms were very similar to those caused by *Pseudomonas solanacearum* (Plate 17), a disease previously not recorded in tomatoes in New Zealand. Drops of exudate containing masses of bacteria oozed from the plants when they were given high humidity (Plate 18). Bacteria in the drops were isolated to King's B Medium agar and to Tetrazolium Medium (Appendix I). The Tetrazolium Medium detects virulent and avirulent

isolates of *P. solanacearum.* Both virulent colonies, large, irregular, hydrous colonies with pink centres, and avirulent colonies, small round deep red colonies (Tuite 1969) were obtained.

Isolates were tested for pathogenicity to tomatoes by watering healthy tomato plants with the bacterial suspension and by injecting the bacterial suspension into the plant stem with a fine gauge hypodermic needle. Some plants were watered with the hydroponic nutrient solution (ex Whangarei) which was suspected of harboring the pathogen. All plants were incubated at 30C because disease development is favoured by high temperatures (Galleghy and Walker 1949). Bacterial wilt symptoms developed in all inoculated plants.

The disease has seldom been a problem in New Zealand as the temperatures are usually not high enough to favour disease development. The disease may have been a problem in the glasshouse at Whangarei because the hydroponic nutrient solution, containing some pathogenic bacteria, was warm enough to favour disease development and also favoured rapid spread of the disease. The pathogen is recorded as being seedborne in some hosts (Neergaard 1977) but there are no reports of it being seedborne in tomato. If the disease was seedborne in tomato then the seed could be the source of primary inoculum for this disease outbreak. *P. solanacearum* is not referred to in Staunton and Cormican's (1978) paper on tomato pathogens in hydroponic systems.

Although temperatures in New Zealand do not favour this disease, all isolates, infected plants and equipment contacting the pathogen in this work was autoclaved at 15 p.s.i. for 15 minutes. The bacteria was inactivated by autoclaving to prevent the disease spreading to other glasshouse plants at Massey University.

Plate 17: Wilted tomato plants resulting from infection by *Pseudomonas solanacearum*.
Bottom right: control plant

Plate 18: Bacterial exudate from tomato plants infected with *Pseudomonas solanacearum* after exposure to a high relative humidity.

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