Epidemiological, genetical, and physiological studies of the bacterial leaf streak pathogen Xanthomonas campestris pv. translucens (J.J.R.) Dowson
by Hee Kyu Kim

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Plant Pathology
Montana State University

Abstract:
Bacterial leaf streak of barley caused by Xantkomonas campestris pv. translucens has occurred on occasion with severity in irrigated barley and wheat fields in Montana. These studies were undertaken to better understand this pathogen in terms of epidemiology, physiology of parasites and genetics.

A first goal was to develop a selective medium useful for isolation of this pathogen from diseased plant materials and other environments. The medium described herein has high selectivity against Pseudomonas syringae from cereals, and against soil-borne phyto-pathogenic and saprophytic bacteria. This medium may prove useful in the epidemiological studies and survival studies of this pathogen.

This is the first report of this pathogen as an active ice nucleator. Only two other microorganisms, Pseudomonas syringae and Erwinia herbicola, also plant associated bacteria, are known to be ice nucleators. This finding suggests that a new mode of transmission is possible and also points to a useful taxonomic differentiation of the "translucens" groun from the "campestris" group.

A cultivar Oderbrucker (C.I. 4666) described to be resistant 50 years ago, is still resistant to the current field pathogens, Xanthomonas campestris pv. translucens, in Montana, indicating that resistance can be relatively stable as compared to resistance in some other host-parasite systems. Results of greenhouse and field inoculation appear to have no correlation. This may be because this pathogen requires specific environmental conditions, such as frost injury or low temperatures with high moisture, in order for a severe epidemic to occur.

The method of transferring transposon from Escherichia Coli to Xanthomonas campestris pv. translucens is described. Transposon mutagenesis is a useful tool to identify the gene for any particular traits, including traits conferring virulence, etc. Transposon may be used to mark strains of bacterial plant pathogen with antibiotic resistance for epidemiological studies.

In dedication to:

my mother and late father.

EPIDEMIOLOGICAL, GENETICAL, AND PHYSIOLOGICAL STUDIES OF THE BACTERIAL LEAF STREAK PATHOGEN *XANTHOMONAS CAMPESTRIS* PV. *TRANSLUCENS* (J. J. R.) Dowson

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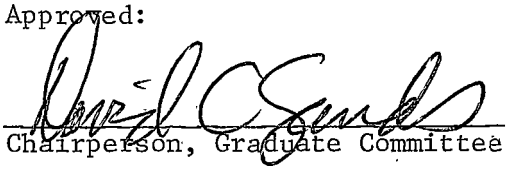
Approved:



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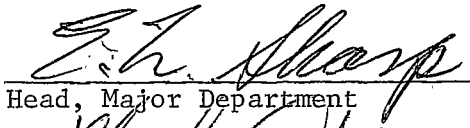

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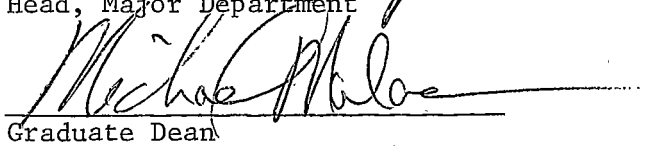

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ABSTRACT

Bacterial leaf streak of barley caused by *Xanthomonas campestris* pv. *translucens* has occurred on occasion with severity in irrigated barley and wheat fields in Montana. These studies were undertaken to better understand this pathogen in terms of epidemiology, physiology of parasites and genetics.

A first goal was to develop a selective medium useful for isolation of this pathogen from diseased plant materials and other environments. The medium described herein has high selectivity against *Pseudomonas syringae* from cereals, and against soil-borne phytopathogenic and saprophytic bacteria. This medium may prove useful in the epidemiological studies and survival studies of this pathogen.

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INTRODUCTION

The plant pathogenic bacteria are important economically because they can cause serious disease losses. A study of one of these pathogenic bacteria is of value if it elucidates any aspect of the pathogen that might permit control of the disease. Additionally, the study of bacterial plant pathogens can aid in the study of basic disease mechanisms. In the well documented cases of *Agrobacterium tumefaciens*, *Pseudomonas savastanoi*, *Corynebacterium sepedonicum*, *Pseudomonas syringae*, these species were chosen to elucidate the basic disease mechanisms of tumor induction, gall formation, toxin production and ice nucleation. In this study, the pathogen *Xanthomonas campestris* pv. *translucens* is a fitting choice for study of host specificity phenomena because some of these xanthomonads are host-limited to a single plant species, while others attack two or more species of hosts. Host range differences are distinct and the host specificity of the cultures of this pathogen is a very stable characteristic. It is the purpose of this study to develop an essential tool for epidemiological studies of this pathogen, to investigate new and unique traits of this bacterium and to develop the technique necessary for genetic analysis of its virulence and its host specificity.

Bacterial leaf streak of barley was first observed in 1912 by

Jones, Johnson and Reddy (1917) and the causal agent, after several nomenclatural changes, is now classified as *Xanthomonas campestris* pv. *translucens* (Jones, Johnson and Reddy) Dowson (1939). This pathogen includes a pathover that infects 17 species of the family Gramineae, although no one strain will attack all 17 species. Geographical distribution of this disease is world-wide.

In Montana, this disease has occurred severely but sporadically in irrigated barley and wheat fields and less occasionally on dryland cereals during wet years. This disease is seed-borne and thus, seeds may serve as a primary source of inoculum, even though it is also dissesminated by rainsplash, or wind-driven rain. The bacteria invade plants through the stomata and hydathodes and through wounds.

Plant pathogenic bacteria are often easily isolated from diseased material, however, rapid growth of saprophytic bacteria can obscure the slow growing pathogens, especially when the pathogens are in low numbers. To date, there has been no reliable method for specifically detecting the bacterium when it is greatly outnumbered by saprophytes or by other pathogens. A medium that only permits the growth of this bacterium, excluding all other microbes, would be useful for epidemiological studies and the identification for the causal organism of disease. A few media have been described for isolation of various xanthomonads, but none has yet been useful for

Xanthomonas campestris pv. *translucens*. The medium employed for isolation of this pathogen is Wilbrink's agar (Dowson, 1957), which is not considered to be selective. Therefore, a selective medium designated as KM-1 was developed for isolation of this organism.

This is the first report of *Xanthomonas campestris* pv. *translucens* as an ice nucleating active agent. It is only the third bacterial genus found to be active in ice nucleation, following the discoveries of this property in *Pseudomonas syringae* and *Erwinia herbicola*. With this disease, epidemics appear to be sporadic. The rate of seed-borne transmission is relatively low, with transmission from less than 2 percent of seeds from highly infected plants. An unknown factor in the field may raise these transmission frequencies to result in an epidemic. The data from this study hint that ice nucleation may be a major mechanism by which these bacteria enhance infection leading to severe epidemics.

A transposon is a useful genetic tool to find and to identify the genes that encode any particular traits such as virulence, etc. Transposons are discrete sequences of DNA that are incapable of self-replication and can be inserted into DNA replicons, chromosomes or plasmids. Such insertions of transposons are random events and, in effect, are strongly mutagenic.

In this study, the method by which the transposon can be

transferred from *Escherichia coli* strain 1830 to *Xanthomonas campestris* pv. *translucens* is described.

LITERATURE REVIEW

The genus *Xanthomonas* Dowson 1939 is defined as Gram negative, polarly flagellated, rod-shaped, phytopathogenic bacteria, the colonies of which are distinctively yellow in color (Dowson 1939). All the *Xanthomonas* species recognized at present are plant pathogens and, so far as is known, are found only in association with plants or plant material (Dye and Lelliott 1975). By 30 different cultural, physiological, and biochemical tests, the 209 phytopathogenic *Xanthomonas* cultures comprising 57 recognized species formed a remarkably uniform group which could be easily distinguished from some other yellow pigment producing organisms that were included for comparative purposes (Dye 1962). Therefore it has been suggested that many *Xanthomonas* species could well be regarded as a single species comprising special forms of one species adapted to particular hosts (Dye 1962). With the exception of four of the nomenspecies, *Xanthomonas fragariae*, *X. albilineans*, *X. axonopodis* and *X. ampelina*, all other xanthomonads can be distinguished only from the type species and/or each other by their host range. These other xanthomonads may be regarded as members of a single taxospecies, referred to as the *Xanthomonas campestris* group (Dye 1980, Dye and Lelliott 1975). Young et al. (1978) proposed that all strains except these four nonemspecies be classified as pathovars of *X. campestris*. All

strains have the following characterisitcs: Gram negative rods 0.4 x 1.0 um, single polar flagellum, aerobic, catalase positive, H_2S positive, hypersensitive reaction to tobacco leaves, oxidase negative, hydrolyse Tween 80, nitrite not produced from nitrate, and indole not produced (Dye 1962, 1980, Dye and Lelliott 1975). Most xanthomonads produce "Gum Xanthan", an extracellular polysaccharide slime produced on media containing glucose. Colonies of these species are mucoid, convex, and shiny on nutrient agar or YDC agr (Dye 1980). The pigment from xanthomonads is a non-water soluble carotenoid which in petroleum ether has an absorption spectrum with maxima at 418, 437 and 463 nm (Starr 1977). A few xanthomonads are non-pigmented, i.e., *X. campestris* pv. *manihotis*, or are known to have non-pigmented forms, i.e., *X. campestris* pv. *ricinii*. These pathovars are indistinguishable from *X. campestris* pv. *campestris* except by host reactions. Two varieties of the nomenspecies, *X. phaseoli*, were only distinguishable by production of a diffusible brown pigment in var. *fuscans* and not in var. *vignicola*. Both are now included in *X. campestris* pv. *phaseoli* with no taxonomic differentiation (Dye and Lelliott 1975). To date, 110 nomenspecies of *Xanthomonas* have been characterized and described as a pathovars of *Xanthomonas campestris* group (Dye and Lelliott 1975) in the 8th Bergey's manual of Determinative Bacteriology. This taxonomic lumping was considered

necessary until further research revealed substantive physiological and biochemical differences among pathovars.

Bacterial blight of barley, now generally referred to as bacterial leaf streak of barley, has been recognized as early as 1912 as a widespread disease capable of causing economic loss. In 1917 Jones, Johnson, and Reddy described the causal organism, *Bacterium translucens*, as follows: cylinderical rods rounded at ends, solitary or in pairs; individual rods 0.5 - 0.8 by 1 - 2.5 um, motile by a single polar flagellum; aerobic, no spores, Gram negative and forming yellow colonies on peptone beef agar plates. Inoculation with this bacterium resulted in infection of barley, but negative results were obtained from inoculations on oats, rye, wheat, spelt, emmer, einkorn, and timothy (Jones, Johnson, Reddy 1917). In 1919 Smith, Jones and Reddy described *Bacterium translucens* var. *undulosum* as a causal agent of the black chaff disease of wheat. It is also known to infect spelt, barley, and rye by artificial inoculation. In 1924 Reddy, Godkin, and Johnson described *Bacterium translucens* var. *secalis* as a causal organism of bacterial blight of rye infecting rye only, but otherwise identical to the above two bacteria. In 1936, Hagborg transferred *B. translucens* var. *undulosum* to the genus *Phytomonas* Bergey et al. and changed its rank to that of a forma specialis, in conformity with the International Rules of

Botanical Nomenclature of 1930. In 1939, Dowson transferred the original species to his newly created genus *Xanthomonas*, as X. *translucens* (Jones, Johnson, and Reddy) Dowson. In 1942, Hagborg revised the classification and amended the species description of X. *translucens* to include the five closely related organisms, which are distinguishable chiefly by differences in pathogenic capabilities on wheat, oats, barley, and rye. One of the organisms, X. *translucens* (Jones, Johnson, and Reddy) Dowson, was given new rank as X. *translucens* f. sp. *hordei*; another, *Phytomonas translucens* f. sp. *undulosa* (Smith, Jones, and Reddy) Hagborg, was transferred to the genus *Xanthomonas*; and the third, *Phytomonas translucens* var. *secalis* (Reddy, Godkin, and Johnson) Bergey et al., was transferred to the genus *Xanthomonas* as X. *translucens* f. sp. *secalis*. One of the two of his new forma specialis could infect barley and oats, but not wheat and rye; and thus was named X. *translucens* f. sp. *hordei-avenae*; the other could infect wheat, oats, barley, rye and was named X. *translucens* f. sp. *cerealis*.

In 1945, Wallin and Reddy reported the forma specialis *phleipratensis* from timothy. Bamberg (1936) suggested the existence of strains of the organism differing in pathogenicity. Hagborg (1942) noticed the difference in pathogenic capabilities occurs between different isolates of the same forma specialis of X. *translucens* and suggested the need for recognizing races within the special forms.

Hagborg (1942) considered the *Triticum* spp. to be the natural hosts of f. sp. *cerealis*, which is more pathogenic on wheat. However, Wallin (1946) extended the definition of *X. translucens* f. sp. *cerealis* and discussed the existence of the biological races of this forma specialis. He found that *X. translucens* f. sp. *cerealis* from brome grass (*Bromus inermis*) was more pathogenic on this species than on wheat, considering it a distinct race of the redescribed forma specialis.

From pathogenicity tests, *X. translucens* f. sp. *cerealis* and *X translucens* f. sp. *hordei-avenae* of Hagborg (1942) are not considered valid forma specialis by Fang et al. (1950) who found that neither of these two infects oats. These were included respectively with f. sp. *undulosa* and f. sp. *hordei*. The f. sp. *hordei*, *undulosa*, *secalis* and *phleipratensis* maintained their original description (Jones, Johnson, and Reddy 1917, Smith, Jones, and Reddy 1919, Hagborg 1942, Wallin and Reddy 1945). Fang et al. (1950) redescribed *X. translucens* f. sp.*cerealis* as follows: occurs naturally on smooth brome grass and quack grass; produces water-soaked streaking symptoms on barley, rye, wheat, smooth brome grass and quack grass following wound inoculation, but it is more virulent on grasses. It will infect smooth brome grass, quack grass, and barley by spraying, but with the same inoculation methods it only slightly infects rye and wheat. They found that the f. sp. *hordei* and *phleipratensis* are serologically distinct with intermediate types of f. spp. *undulosa*, *secalis*, and

phleipratensis are serologically distinct with intermediate types of f. spp. *undulosa*, *secalis*, and *cerealis*. Attempts to use form specific phages to identify cultures of X. *translucens* were unsuccessful (Katzelson and Sutton 1953). Moffett and McCarthy (1973) found X. *translucens* isolates from Japanese millet to be less pathogenic on wheat than on the originally recorded host and suggested the possibility of distinct races of X. *translucens* f. sp. *cerealis*. Bradbury (1971) proposed the nomenclature of bacterial streak pathogen of rice (*Oryza sativa* L.) as *Xanthomonas translucens* f. sp. *oryzicola*, a pathotype originally isolated from rice which by artificial inoculation infects only rice.

Six pathotypes of X. *translucens* using forma specialis designation are accepted in the 8th Bergey's manual of Determinative Bacteriology, using the criteria of production of translucent, water-soaked lesions on unwounded plants and the data of Fang, Allen, Riker, and Dickson (1950), Tominaga (1967) and Patel and Shekehwat (1971). The "translucens" group can well be regarded as a large group comprising a number of pathotypes, some with overlapping host ranges within this nomenspecies. These pathovars are detected at the genus level of host specificity. Variations among cultivars have not been analyzed in this way.

Culturally, physiologically, and biochemically indistinguishable

from *X. campestris* except by host range, this pathogen has been recently named a pathovar of the *X. campestris* group, *Xanthomonas campestris* pv. *translucens* (Dye 1962, 1980, Dye and Lelliott 1975, Young, Dye, Bradbury, Panagopoulus, and Robb 1978).

Etiology

The seed-borne nature of this disease was reported by Jones, Johnson, and Reddy 1916, Smith, Jones, and Reddy in 1919. The bacterium penetrates the pericarp causing infection of the plumule through wounds or stomata on the coleoptile. Spreading rapidly through these tissues, it finally reaches the enclosed foliage leaves. By elongation the infected leaf carries the bacteria into the aerial parts of the seedling with water soaked streaks on the primary leaf (Wallin, 1946).

The bacterium infects leaves through stomata, spreading intercellularly (Jones, Johnson, and Reddy 1917, Bamberg 1936). The bacterial invasion is confined only to the thin-walled parenchyma possessing intercellular space, i.e., mesophyll of leaf, chlorenchyma and ground tissue parenchyma of the leaf sheath. In naturally infected leaves, no vascular bundle elements were shown to be infected (Shekhawat and Patel 1978).

This disease was described as a widely occurring disease attacking leaves, leaf sheaths and glumes, early characterized by

water-soaked lesions with bacterial exudates, and later by persistent transparency following the death of the parts invaded. The sporadic occurrence of infection may have been due to the variation in environment. Bamberg (1936) found that low relative humidity of the surrounding atmosphere retarded the growth of the bacterium in culture when the relative humidity was maintained with aqueous solution of sulphuric acid. This retarding effect was noticeable at a relative humidity of 50 percent and was greater as the percentage lowered. The optimum temperature for the growth of this organism on agar has been reported to be in the range of 25 - 30 C (Bamberg 1936), and 24 C (Stewart 1952). The maximum temperature for sustained growth is approximately 40 C with the minimum slightly below 10 C (Bamberg 1936).

The organism is very resistant to dry conditions, surviving for eight months on straw from blighted plants and two years on seed (Jones, Johnson, and Reddy 1917) and 77 days in dry soil (Bamberg, 1936). Moreover, this organism is also resistant to extremely low temperatures ranging from -33 C to 30 C, remaining viable in soil cultures for at least 124 days after December 15, at St. Paul, Minnesota (Bamberg, 1936). On the other hand, Boosalis (1952) reported that *X. translucens* does not survive in non-sterile soil for an appreciable length of time unless it is in or on plant parts. This organism may persist from one season to the next in

seed of barley and wheat, on straw of barley, brome grass and other winter hosts.

Host Range and Epidemiology

The host range encompassed by this translucens group has been reported to include 17 different hosts in the Gramineae: barley (*Hordeum vulgare* L.), foxtail barley (*Hordeum jubatum* L.), wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), spelt (*T. spelta* L.), Einkorn (*T. monococcum* L.), victory oat (*Avena sativa* L.), smooth brome (*Bromus inermis* Leyss.), cheatgrass brome (*B. tectorum* L.), yellow bristle grass (*Setaria lutescens* (Weisel.) F. T. Hubb.), quackgrass (*Agropryon repens* (L.) Beauv.), orchardgrass (*Dactylis glomerata* L.), water foxtail (*Alopecurus geniculatus* L.), reed canary-grass (*Phalaris arundinaceae* L.), rice (*Oryza sativa* L.) Japanese millet (*Echinochloa crus-galli* var. *frumentaceae*.), and timothy (*Pleum pratense* L.). These studies are referred to in Table 1 of Appendix.

Considering the wide host range data and the epidemiological studies cited above, this pathogen may overwinter on diseased stubble or refuse or on overwintering hosts, becoming a source of spring infection.

The most severe epidemics of this disease occur in relatively warm, wet seasons with the optimum temperature at 20 C. The size of lesion is limited and symptom development is delayed at or below

10 C (Bamberg, 1936). Stewart (1952) observed highest infection at 15.5 - 18 C, moderate infection at 21 C and slight infection at 24 C,with no infection at 27 C, an optimum temperature for growth in culture. A shift of about 5 C cooler than the optimum temperature in culture is more conducive for symptom development. The low relative humidity is probably more often a limiting factor in the development of disease than is low temperature (Bamberg, 1936). Effective transmission of aphids, straw, wind and rain, and by diseased plants coming in contact with healthy plants, occurred only when the host was water-congested (Boosalis, 1952). Field epidemiology studies in Montana using a marked (antibiotic resistant) strain of *Xanthomonas translucens* f. sp. *hordei* indicated that this bacterium is capable of spreading 28 square meters from a single infection locus within 39 days (Hall, Kim, and Sands, 1981).

The distribution of this disease occurs where barley or cereals are grown. *Xanthomonas translucens* was reported as early as 1917 in Colorado, Iowa, Minnesota, Montana, North Dakota, South Dakota, Oregon, Wisconsin and Ohio in the United States (Jones, Johnson, and Reddy), and in Canada, Mexico, Russia, France, Belgium, and Africa (Bamberg, 1936).

Screening of disease resistance

Techniques used for inoculation into cereals include 1) spraying

the bacterial suspension (Jones, Johnson, and Reddy 1917, Fang et al. 1950, Stewart 1952), 2) partial vacuum technique (Boosalis, 1952), and 3) hypodermic introduction of bacterial suspensions by hypodermic syringe (Bamber 1936, Hagborg 1936, Fang et al. 1950). After the hulled barley seed is soaked in bacterial suspension for one hour, 54 percent of the plant is infected. At least 24 hour soaking is needed for non-hulled seeds to yield 50 percent diseased seedlings (Wallin, 1946). Seed and seedling inoculation methods, unaccompanied by wounding, proved to give unsatisfactory results (Bamberg 1936, Hagborg 1936). The inoculation method of forcing inoculum into the leaf roll of young plants or into the leaf sheath of the older plant with hypodermic syringe is generally thought to be the most successful (Bamberg 1936, Hagborg 1936, Fange et al. 1950, Wallin 1946). Hagborg (1936) developed an inoculation method involving minimum injury to the coleoptiles and the enclosed primary leaves. The seedlings were poked with a sharp nichrome needle when they were only 7 - 15 mm above the soil. Then the seedlings were flooded with the bacterial suspension, placed in the greenhouse at 25 C for ten days, and examined for infection.

For an inoculation method to be useful, it must 1) closely simulate natural infection, and 2) result in symptoms closely resembling those that occur in the field. Stewart (1952) reported the barley cultivars, Spartan and C. I. 6240, showed high degrees of

resistance in the field but less under artificial inoculation in the greenhouse. Bamberg (1936) reported that the wheat varieties, Marquis, Mindum and Kubanka, which appeared resistant to natural infection in the field, were rather severely infected when hypodermically inoculated. He suggested that some of the resistance to black chaff under natural condition may be morphological or functional, as it is apparently in some cases of resistance of wheat to stem rust. This type of resistance may be eliminated when hypodermic inoculations are made. Prerequisite to determining resistance are proper inoculation techniques that give reproducible results coinciding closely with field observations.

Chevalier, California, Summit and Oderbrucker (C.I. 4666) were reported to be the most resistant of 53 varieties tested (Jones, Johnson and Reddy 1917). This cultivar Summit is six-rowed and is different from the cultivar Summit recently released. In the epiphytotics of 1944, Oderbrucker (C.I. 4666), a Chevron (C.I. 1111) x Bolivia (C.I. 1257) selection and many selections of the Manchuria germplasm group showed resistance (Dickson, 1956).

Waldron (1929) found an average of 10.5 percent decrease in 1,000 kernel weight of several F_3 families from crosses of susceptible variety Hope with other varieties, when these plants were exposed to infection.

Chemical control

Seed treatment for two hours in formaldehyde solution eliminates the organism from the seed; hot water treatment is also recommended for control of this disease (Jones, Johnson, and Reddy 1917). Mergamma, Agrox, Ceresan, and streptomycin were tested for their effectiveness in controlling this disease. Mergamma gave the highest level of control, however, no chemical treatment eliminated the bacterium completely (Stewart, 1952). Streptomycin was least effective and reduced plant stand counts. Streptomycin and chloramphenicol added to the culture solution increased the resistance of spring wheat Thatcher (C.I. 10003) seedling grown in Shive's four-salt solution with trace elements added (Hagborg, 1956). Reduction in the dry weight per plant by antibiotics was noted, but the resistance of plants to *X. translucens* was obtained apparently by absorption of antibiotics through the root system.

This pathogen is very susceptible to mercurials, and therefore seed treatment with mercuric chloride has been recommended along with crop rotation, field sanitation, clean seed for control (Dickson 1956, Jones, Johnson, and Reddy 1917). The registration and use of mercuric chloride and most organic mercurials has been cancelled. The bactericide cupric hydroxide (Kocide Chemical Corp. Houston,

Texas) has recently been registered and recommended for seed treatment, without evidence of complete control.

Selective Media

A few media have been described for various xanthomonads (Kado and Heskett 1970, Mulrean and Schroth 1981, Schaad and White 1974), but none has been useful for isolation of *Xanthomonas campestris* pv. *translucens*. The medium commonly employed for isolating this pathogen is Wilbrink's agar (Dowson, 1957) which is not considered to be selective. SX agar was described in 1974 by Schaad and White for isolation of *Xanthomonas campestris* from soil. A semi-selective medium for isolation of *Xanthomonas campestris* pv. *juglandis* from walnut was developed by Mulrean and Schroth in 1981. Kado and Heskett in 1970 reported that their D-5 medium selectively favors the growth of *Xanthomonas* spp. and *Agrobacterium tumefaciens* while suppressing the growth of *Pseudomonas* spp.

Ice nucleation

The initiation of precipitation over much of the earth's surface is believed to be caused by the development of ice nucleation in supercooled clouds (Schnell and Vali, 1973). The source, composition and number of small particles initiating freezing events in natural clouds are poorly understood. Ice nuclei are defined as particles initiating the phase transition to ice at temperatures

between 0 and -40 C (Schnell and Vali, 1972). These particles represent a very small fraction, i.e. only one part in 10^{10} - 10^{12} of the total aerosol mass in the atmosphere (Vali and Schnell, 1975).

In 1964, Panagopoulos and Crosse determined frost injury to be a predisposing factor for the development of the symptom of blossom blight of pear caused by *Pseudomonas syringae*, which was found to be present on the leaves and blossoms of pear. In 1974, Sands and Kollas reported *Pseudomonas syringae* to be pear blast causal agent in Connecticut. This disease was reported to have appeared under unusually moist weather conditions at the time of pear blossoming. Typical symptoms were developed after 5 to 6 days of lower temperatures in the -2 C or slightly below during the greentip to one-half inch green period of development, even without frost. Since these discoveries, aerobiologists started to pay attention to the possible role bacteria has in the initiation of precipitation

Recent research has shown that at least the potential exists for biological materials to contribute significant proportions of atmospheric ice nuclei. The transient appearances of ice nuclei active at temperatures of -2 to -5 C had been noticed to accompany the natural decay of the plant leaf material (Schnell and Vali, 1972). In a preliminary attempt to isolate the entity responsible for increased frost damage, Arny et al. (1976) found that water extracts of dried powdered corn leaves caused increased frost sensitivity

when applied as a spray to seedling corn. Addition of either streptomycin or tetracycline at 1,000 ppm to those water extracts eliminated their activity, suggesting the possible role of bacteria in this effect.

In recent years a suggestion was proposed that biogenic ice nuclei, such as leaf-derived nuclei, may constitute a significant fraction of the atmospheric ice nuclei (Schnell and Vali, 1972, 1973, 1976, Vali et al., 1976). Viable bacteria were found from marine fogs and were proven to exhibit ice nucleation activity at temperatures of -4 C in sterile sea water and -2 C in distilled water (Carney, Schnell and Carthy, 1975). Later it was shown that the development of these nuclei results from the worldwide ubiquitous presence of a bacterium *Pseudomonas syringae* (Schnell and Vali 1973, Vali et al. 1976). The evidence of *Pseudomonas syringae* as biogenic ice nuclei and its significance in epidemiology of bacterial blight was further substantiated by Sands, Langhans, de Smet, and Scharen (1981) who isolated this pathogen from raindrops in rainstorms at an elevation from 180 meters to 1,800 meters above the cropland in Montana. These findings give possible meterological implications to the interactions between bacteria and rain. *Pseudomonas syringae* van Hall has been identified as an epiphyte and occasional pathogen (Dowler and Weaver 1975, Panagopoulos

and Cross 1964) and found to be responsible for increased frost sensitivity of corn, bean or lettuce at -5 C or above, while no strains of the *Pseudomonas tabaci*, *P. glycinea*, *P. phaseolicola*, *P. marginalis*, *Erwinia stewartii*, *Escherichia coli* were active (Arny, Lindow, and Upper 1976, Lindow et al, 1975).

Lindow et al. (1978) identified another ice nucleation active bacterium, *Erwinia herbicola* (Loehnis) Dye, which is also active in promoting frost injury of frost sensitive plants at -2.3 C. and below when sprayed with suspension of 10^7 colony forming units (CFU) per milliliter. Both *Pseudomonas syringae* and *Erwinia herbicola* are commonly present as epiphytes on a diversity of plant species from widely separated areas of the United States (Lindow et al, 1975, 1978). Sufficient numbers of ice nucleation active bacteria were present on the leaves to account for the freezing injury to occur (Lindow et al., 1978).

Many species of several genera of phytopathogenic bacteria were tested to determine their ability to act as ice nuclei (Arny et al. 1976, Hirano et al. 1978, Paulin and Luisetti 1978). Positive results have only been obtained with *Pseudomonas* strains, and with one exceptional strain of *Erwinia herbicola* out of about 400 strains. Of 21 strains of *Xanthomonas campestris* pv. *juglandis*, none were ice nucleators. Single strains of *X. campestris* pv. *vitians* and *X. campestris* pv. *maculifoliigardeniae* were also tested negative for

ice nucleation (Paulin and Luisetti, 1978). Attempts were made to use this ice nucleating activity as a taxonomic tool in conjunction with other biochemical and physiological tests (Hirano et al., 1978).

Application of antibiotics such as streptomycin and tetracycline eliminated the ice nucleation activity and prevented the sensitive plants from frost injury (Arny et al. 1976, Lindow et al. 1975). The effects of dyes, antibiotics, and chemical agents on ice nucleating activity of *Pseudomonas syringae* were studied extensively by Maki et al. (1974). The dyes such as methylene blue, crystal violet, and safranin, which combine with the cell wall of bacteria, destroyed the cell activity. Conversely, congo red, which does not combine with the cell wall, did not affect the activity. Both mercuric chloride treatments and heat treatments at 65 C for 5 minutes destroyed the activity. The above observations suggest that ice nucleation activity may be cell associated and, further, may be associated with the cell wall.

History of R factor and transposon

Transfer of bacterial genes can result from the mating of fertility (F) factor, from transduction by lysogenized phage and also from transformation. The latter is defined as genetic recombination brought about by the introduction of purified chromosomes. The

fertility factors can exist either as integral parts of chromosomes or as small free circular chromosomes that multiply once per cell division. Only when the F factor is part of the chromosome can the male cells mate. These F^+ cells are called Hfr (high frequency of recombination) (Watson, 1977).

In addition to the sex factors which carry genes directly involved in the mating, most bacteria contain other freely replicating circular extra-chromosomal elements termed plasmids. Many of the common plasmids are known as resistant transfer factors (RTF) or resistant (R) factors that confer simultaneous multiple resistance to many different antibiotics (Watson, 1977).

Two groups of pili, common pili and sex pili, are known in the Enterobacteriaceae. Sex pili are distinguished from common pili by being of longer length, having terminal knobs, occurring in clusters, and serving as phage absorption sites. Conversely, common pili are short, less than 1.5 u in length, and have no terminal knob, clustering or phage adsorption. Most sex pili are characterized into two groups, typified by F and I pili, respectively, by morphology, phage adsorption and antigenic structure (Lawn et al., 1967). Even though there are certain differences between the two groups of sex pili, I-like pili are usually shorter (0.2 um in length) than F-like pili (20.0 um in length). The knobs at the end of F-like pili, tend to be longer and more complex than those of I-like pili, which are

usually small and undifferentiated (Lawn et al. 1967).

Naturally occurring R factors have been classified into two categories, fi^+ (fertility inhibition$^+$) and fi^-, depending on their ability to inhibit F-mediated conjugation (Watanabe et al. 1964). The fi^- R factors have ability to restrict phage multiplication. The fi^+ R factors determine F-type pili (Lawn et al. 1967) and constitute one class of plasmid (Datta and Hedges 1971). However, the classical fi^- R factors were indicated to be heterogeneous (Lawn et al. 1967, Datta and Hedges 1971). Among 26 fi^- R factors, twenty determined the production of I-type pili, supporting the multiplication of I-pili specific phage, while six did not (Lawn et al. 1967). In the latter group, there was no evidence of production of I-pili. Thus fi^- R factors are classified into four plasmid incompatibility groups called I, P, N. and W, based on the superinfection immunity, surface exclusion and incompatibility (Datta and Hedges 1971, 1972, Datta et al. 1971).

The R factor RP1 which originated in *Pseudomonas aeruginosa* 1822 confers resistance to carbenicillin, neomycin, kanamycin and tetracycline and has a molecular weight of about 40 million daltons, 60 percent GC and a buoyant density of 1.719 g/cm^3 (Grinsted et al. 1972). RP4, an R factor derived from *Pseudomonas aeruginosa* strain S8 and specifying resistance to carbenicillin, neomycin, kanamycin and tetracycline, was mediated by a plasmid of molecular weight about

62 million and the buoyant density 1.710 g/cm^3, 60 percent GC (Saunders and Grinsted, 1972). RP1 and RP4 are very similar to each other by base sequences (Saunders and Grinsted, 1972). RP4, a p-group plasmid, proved to have exceptionally wide host range (Datta et al. 1971, Datta and Hedges 1972) comprising *Escherichia, Salmonella, Shigella* and Proteus group and *Rhizobium* spp. *Agrobacterium* spp. P-group plasmids have been transferred by conjugation from *Pseudomonas aeruginosa* to the plant associated *Rhizobium* spp. (Boucher et al, 1977), plant pathogenic *Erwinia* spp. (Cho et al. 1975), *Agrobacterium tumefaciens* (Levin et al. 1976), *Xanthomonas vesicatoria* (Lai et al. 1977) and also to the phytopathogenic pseudomonads *P. glycinea, P. pisi, P. phaseoloicol* and *P. syringae* (Panopoulos et al. 1975, Lacy and Leary 1976). Plasmid transfer between physiologically capable cells in plants is not limited by the host nor does it require a phytopathogen to phytopathogen relationship (Lacy and Leary 1975).

The wide host range of P-group plasmid implies important economic and epidemiological significance of antibiotic resistance plasmids in the control of plant disease. Recently, Jacob and Grinter (1975) reported that Plasmid RP4 has only one site susceptible to cutting by the restriction enzyme EcoR1. Therefore the P-group plasmid could be a useful tool for genetic study of phytopathogenic bacteria (Boistard and Boucher 1978).

Drug-resistance elements are translocatable, termed transposons and are discrete sequences of DNA incapable of self-replication at a random location. In order to be replicated, it must be inserted into DNA replicons, such as chromosomes or plasmids. When it occurs in a structural gene, the result is generally non-leaky polar mutations (Kleckner et al. 1977). Recently Boucher et al. (1977) reported that when the lysogenic phage Mu was inserted into RP4, the transfer frequency of plasmid from *Escherichia coli* to *Rhizobium meliloti* was reduced from 3×10^{-4} to 2×10^{-9}, about 10^5-fold, possibly due to the suicidal effect of the phage leading to the destruction of the plasmid. Van Vliet et al. (1978) showed that the same principle applies to transfer from *Escherichia coli* to *Agrobacterium tumefaciens*. Those plasmids which do establish in this new host have large deletion of the Mu phage. They reported that the instability of the RP4::Mu system could be utilized to carry transposons into this species to obtain transposon mutagensis. Taking advantage of this suicidal effect of Mu phage, causing inability of Mu-inserted P-group plasmids to become established in recipient cells, Beringer et al. (1978) constructed an *Escherichia coli* strain 1830 harboring a plasmid carrying Mu bacteriophage and the Tn5, transposon coding for kanamycin resistance and also relatively nonspecific in the sites of integration into *E. coli* chromosome (Kleckner et al. 1977). This strain is derived from

a P1 plasmid, pPH1JI, conferring resistance to gentamycin and to very low concentrations of streptomycin; it has a wide host range in which the Tn5 and Mu phage was inserted by Beringer et al. (1978). This strain was found to be Mu immune, unable to produce viable Mu phage. This constructed plasmid was successfully used as a carrier of Tn5 into three *Rhizobium* spp. Owing to the inability of strain K60 of *Pseudomonas solanacearum* to stably maintain P1 plasmid, Boucher et al. (1981) reported that with Tn5 and Tn10 inserted into P1 plasmid, but without Mu phage inserted, he was able to have vehicles which were mutagenic that transposed with relatively high frequencies of ca. 5×10^{-6}. He found that under the same conditions with Tn7 the transposition frequency was low.

By using this transposon that integrates at random, we can determine the frequency of avirulent mutants among the transconjugants. This should permit an approximate estimation of the numerical portion of genome that involves genes for virulence.

Chapter 1

DEVELOPMENT OF A SELECTIVE MEDIUM FOR *XANTHOMONAS CAMPESTRIS* PV. *TRANSLUCENS*

INTRODUCTION

The objective of this study was to develop a selective medium, an essential tool for epidemiological studies of this pathogen. Selective medium development is usually an important step in the scientific study of a bacterial plant pathogen.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this work are listed in Table 1. *Xanthomonas campestris* pv. *translucens*, hereinafter referred to as *X. c. t.* and *Agrobacterium* spp., were maintained on Wilbrink's agar (Dowson, 1957); 10 g of sucrose, 5 g. of Bacto-peptone (Difco Laboratories, Detroit, MI.), 0.5 g. of K_2HPO_4, 0.25 g of $MgSO_4.7H_2O$, 0.05 g. of Na_2SO_3 and 15 g of bacto-agar (Difco) per liter of distilled water. All other strains of *Xanthomonas* were maintained on Nutrient broth (Difco), 5 g of casein hydrolysate (Sigma Chemical Co., St. Louis, MO.), 1 g of yeast extract (Difco), 2.0 g. of K_2HPO_4, 0.5 g of KH_2PO_4, and 15 g of Bacto-agar per liter of distilled water. *Corynebacterium sepedonicum* and *C. insidiosum*

were maintained on yeast glucose calcium carbonate agar; 15 g of glucose, 10 g of yeast extract, 5.0 g of $CaCO_3$, 20 g of agar per liter of distilled water. *Bacillus cereus*, *Erwinia cartovora* var. *carotovora* and *E. carotovora* var. *atroseptica* were maintained on Nutrient agar (Difco Nutrient broth amended with 15 g of Difco agar). *Escherichia coli* was maintained on Luria's agar containing 10 g of casein hydrolysate, 10 g of NaCl, 5 g of yeast extract, 15 g of Bacto-agar per liter of distilled water. *Pseudomonas* spp. were maintained on King's B agar (King et al., 1954). All strains were transferred regularly and stored at 3 - 5 C.

Selective Medium (KM-1)

This selective medium for X. c. t. contains 10 g of lactose (Sigma Chemical Co., St. Louis, MO.), 4.0 g. of D(+) trehalose (Sigma), 0.2 g of thiobarbituric acid (Sigma), 0.8 g of K_2HPO_4 and KH_2PO_4 (Sigma) respectively, 0.03 g of yeast extract (Difco Laboratories, Detroit, MI.), 1 g of NH_4Cl (Mallinckroft Chemical Works, St. Louis, MO.) and 15 g of Bacto agar per liter of double distilled water. Before adding agar, the ingredients were dissolved completely on a hot plate with stirring bar and pH was adjusted to 6.6 with 1 N NaOH solution. After autoclaving these ingredients, cycloheximide (Sigma), dissolved in ethanol, tobramycin (Sigma), dissolved in ethanol-water (1:1, v/v), and ampicillin (Sigma), dissolved in

ethanol-water (1:1, v/v) with addition of 1 pellet of sodium hydroxide (Mallinckroft Inc., Paris, Kentucky) were added aseptically to the final concentration of 100 ug/ml, 8 ug/ml and 1 ug/ml, respectively.

Choice of components of KM-1

Dowson (1939) reported that all the species of his newly created genus *Xanthomonas* utilized lactose as a carbon source. Thus, lactose is one of the carbon sources that appears to be promising as a selective medium component for *Xanthomonas*. Trehalose, a four carbon sugar, is also a good nutritional substrate for the *Xanthomonas campestris* group (Dye and Lelliott 1975). *Pseudomonas fluorescens* also utilizes trehalose (Misaghi and Grogan 1969, Stanier et al. 1966), but is not used by any of the members of the *Pseudonomas syringae* group, *Pseudomonas cichorii* (Sands et al., 1980), or *Pseudomonas putida*, or *Pseudomonas aeruginosa* (Stanier et al. 1966). The combination of these carbon sources provides selectivity against *Pseudomonas syringae*, which has been recognized as a common contaminant in the isolation of *X. c. t.* from diseased material ever since 1936 (Hagborg). Thiobarbituric acid is another component of selective value. Ammonium chloride as a nitrogen source is useful in enhancing the yellowish colony color, a key visual characteristic of these xanthomonads. Yeast extract is a necessary

source of nutritional enrichment, permitting these xanthomonads to grow at reasonably fast rates. The basal medium of KM-1 without antibiotics, exhibits fair selectivity against the fluorescent, oxidase negative, "syringae group" of pseudomonads from cereals.

The key problem in developing the selective medium for *X. c. t.* was that saprophytes grow more rapidly and are, in fact, more resistant to most antibiotics. Most plant pathogenic pseudomonads and other plant pathogenic bacteria grow faster than this pathogen in most of the media. We found that fast growing contaminant bacteria can be suppressed by using diluted concentrations of either penicillin or ampicillin, however, this pathogen is more tolerant of ampicillin than penicillin. In both cases, the mechanism of action of the above two antibiotics inhibits actively growing cells. A search for several other antibiotics with different modes of action was initiated. Tobramycin was found very useful to suppress the fast growing pseudomonads, especially the ubiquitous saprophytic fluorescent, oxidase-positive, nutritionally versatile pseudomonads relatively resistant to other antibiotics. Cycloheximide, a broad spectrum anitfungal agent, was also added.

Characterization of bacterial isolates from the KM-1 selective medium.

Color, shape, and form of colony were initially used to distinguish *X. c. t.* Selected colonies were also transferred to Wilbrink's

agar to observe the rapid development of yellow, mucoid growth, which characterize xanthomonads. These colonies were further confirmed by oxidase test and pathogenicity tests.

Relative colony development of *Xanthomonas campestris* pv. *translucens*.

Single colony culture from Wilbrink's agar was suspended in 5 ml phosphate-buffered saline (PBS) containing 0.85 percent NaCl, 0.57 percent K_2HPO_4 and 0.34 percent KH_2PO_4 (pH 6.8), after adjusting cell suspensions to contain approximately 10^6 colony forming units (CFU) per milliliter as determined by a Klett-Summerson photometric colorimeter with the green filter (Model 800-3, Klett Mfg. Inc., NY., U.S.A.). The serial ten-fold dilutions were made on PBS. Using a spinning turntable, one-tenth of a milliliter of diluted cell suspensions was spread with an L-shaped glassrod over the surface of KM-1 medium and Wilbrink's medium in each of three plates. Plates were incubated at 28° C for 5 - 7 days. Unless otherwise stated, the dilution plating procedure and incubation period are the same throughout this report.

Relative colony development of other *Xanthomonas* nonemspecies

Some strains of *Xanthomonas* spp. other than *X. c. t.* do not grow very well on Wilbrink's agar. Therefore, the relative colony development on KM-1 medium was also compared with nutrient agar

amended with 1 percent glucose.

Spectrum of inhibition

Isolates were first replica plated, and then dilution plated to determine the spectrum of inhibition of this KM-1 medium. The isolates that grew poorly, if at all, on the KM-1 medium by replica plating were selected. Dilution plating was carried out to see how selective this medium was to those bacteria (Table 1).

In order to determine the spectrum of inhibition to soil borne plant pathogenic bacteria and saprophytic bacteria, three strains of *X. c. t.* were mixed with 11 different nomenspecies of bacteria (Table 5). After the dilution plating method was used, the plates were observed in 2 - 3 days for fast growing organisms and again in 5 - 7 days for *X. c. t.* at 28° C.

Selectivity tests

Selectivity of the KM-1 medium was tested by the dilution plating method of various plant samples, such as 1) old samples naturally infected with *X. c. t.*, 2) fresh samples naturally infected, 3) old barley leaf debris not infected, and 4) soil samples from greenhouse. Old barley leaf debris was rinsed with sterile distilled water to remove soil particles and kept in phosphate-buffered saline (PBS) containing 0.85 percent NaCl, 0.57 percent dibasic potassium phosphate (K_2HPO_4), 0.34 percent monobasic potassium

phosphate (KH_2PO_4), for four hours. Old and fresh samples of naturally infected barley were kept in PBS for the same period of time. No surface sterilization was attempted for any plant materials. The 10 grams of the soil sample from the greenhouse were suspended in 100 ml distilled sterile water and stirred vigorously with a stirring bar for 30 minutes. After 10 minutes settling, the supernatant was centrifuged for 20 minutes at 1,000 g force to remove plant materials and soil particles. The supernatant was centrifuged at 3,000 g for 20 minutes. The pellet was suspended in 3 ml PBS and used as the sample for the dilution plating method in which 0.1 ml of each appropriate dilution on PBS was spread on three replicates of both KM-1 medium and Wilbrink's medium.

Shelf life of KM-1 medium

Several packages of this medium were stored at 5° C in a cold room from one week to two months. The selectivity of these KM-1 media were checked at intervals up to two months by the dilution plating method using strains listed in Table 1.

RESULTS

Relative colony development of *Xanthomonas campestris* pv. *translucens*

The average number of colonies of 11 strains of *X. c. t.* developed on KM-1 medium was from 0.91 to 2.13 times greater than on

Table 1. Strains used for evaluation of medium selective for *Xanthomonas campestris* pv. *translucens*.

Nomenspecies and designation	Source[a]
Agrobacterium tumefaciens	1
Agrobacterium rhizogenes	1
Bacillus cereus	2
Corynebacterium insidiosum	2
Corynebacterium sepedonicum	2
Erwinia carotovora var. *atroseptica*	2
Erwinia carotovora var. *carotovora*	2
Escherichia coli	1
Pseudomonas aeruginosa	5
Pseudomonas fluorescens, Pf5	6
Pseudomonas phaseoli, G 50	7
Pseudomonas phaseoli, HB 20	7
Pseudomonas savastanoi	3
Pseudomonas syringae, (8 strains)	10
Pseudomonas syringae pv. *tagetis*	4
Pseudomonas putida	10
Rhizobium phaseoli, 1233	3
Xanthomonas albilineans, 29184	8
Xanthomonas axonopodis, 19312	8
Xanthomonas fragariae, NCPPB 2473	8
Xanthomonas campestris pv. *begoniae*, 077-3382	8
X. campestris pv. *carotae*, Floral-1	8
X. campestris pv. *campestris*, # 73	8
X. campestris pv. *manihotis*, Xm6	8
X. campestris pv. *pelargonii*, 078-1100	8
X. campestris pv. *phaseoli*, 86	8
X. campestris pv. *pruni*, 8D51	9
X. campestris pv. *translucens*, (11 strains)	10
X. campestris pv. *vesicatoria*, # 26	8
X. campestris pv. *vitians*, 068-790, 7D51	9
068-1406, 7D5	9
069-561, 7D42	9

[a]1 = J. M. Jaynes, 2 = R. Davidson, 3 = R. V. Miller, 4 = B. Simmonds; Dept. of Plant Pathology, MSU, Bozeman, MT., 5 = N. M. Nelson, Dept. of Microbiology, MSU, Bozeman, MT., 6 = C. R. Howell, Univ. of TX, College sta., TX., 7 = S. S. Patil, Univ. of HI, Honolulu, HI., 8 = M. Sasser, Dept. of Plant Sci., Univ. of DE, Newark, DE., 9 = C. I. Kado, UC, Davis, CA., 10 = Author.

Wilbrink's medium (Table 2). Generally, more colonies appeared on the KM-1 medium than on rich and non-selective Wilbrink's medium. The size of colonies was from 1.5 - 2.0 to 3.5 - 4.0 mm in 5 - 7 days with yellowish color. All strains of X. c. *t.* grew well on this selective medium when a loopful of cell suspension was streaked and incubated for 5 - 7 days.

Relative colony development of other *Xanthomonas* nomenspecies.

As shown in Table 3, Nutrient agar plus glucose is a good medium for most *Xanthomonas* nomenspecies, whereas few of those grew on Wilbrink's. Single strains each of *Xanthomonas axonopodis*, *Xanthomonas campestris* pv. *campestris* and X. *campestris* pv. *vesticatoria* grew on Wilbrink's medium with colony sizes from 0.5 - 3.0 mm in diameter. Out of 12 nonemspecies, two strains of X. *campestris* pv. *vitians* 7D5 and 7D52 grew poorly or not at all on the Wilbrink's medium, whereas the strain 7D52 of the same species grew on Wilbrink's medium. However, the number of colonies developed on this medium was about half of those on the Nutrient agar.

Whereas one strain each of *Xanthomonas alibilineans* and X. *fragariae* did not grow on KM-1 medium, one strain each of X. *axonopodis* and X. *campestris* pv. *begoniae* did, even though the plating efficiency of these strains was generally low. One strain out of three strains of X. *campestris* pv. *vitians* did not grow at