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Bacterial bioluminescence: A tool to study host-pathogen interactions between *Brassica oleracea* and the bacterial phytopathogen *Xanthomonas campestris* pv. *campestris* in black rot of cabbage

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BACTERIAL BIOLUMINESCENCE: A TOOL TO STUDY HOST-PATHOGEN INTERACTIONS BETWEEN *BRASSICA OLERACEA* AND THE BACTERIAL PHYTOPATHOGEN *XANTHOMONAS CAMPESTRIS* PV. *CAMPESTRIS* IN BLACK ROT OF CABBAGE

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ABSTRACT

Black rot of cabbage, caused by the bacterial pathogen *Xanthomonas campestris* pv. *campestris*, is a very serious disease, which results in significant crop losses worldwide. Gene technology has provided a unique marker, bacterial bioluminescence, to trace the movement of bacteria in an infected plant and their spread in the field without interrupting the disease process.

Xanthomonas campestris pv. *campestris* strain G-171 was tranformed to the bioluminescent strain 171LIIH-7 in a mating with *Escherichia coli* HB101 harboring plasmid pUCD607. The plasmid carries the genes for bioluminescence and four antibiotic resistance loci. Stability of the plasmid-borne genes as well as expression of pathogenicity and virulence were confirmed in serial passage through agar plates and cabbage seedlings. Southern hybridization patterns and other *in vitro* tests suggest that the genes for bioluminescence and antibiotic resistances are integrated into the chromosome. Growth of the transconjugant at different temperatures and with various nitrogen compounds was compared to that of the wild type strain in liquid culture, and showed that the two strains behave similarly except for a temperature sensitivity for 171LIIH-7 above 30 C.

Once it was determined that the transconjugant stably expressed bioluminescence and pathogenicity, the applicability of the bioluminescence marker to studies of black rot of cabbage was examined. The effect of host nutrition on disease progression and severity was investigated by growing *Brassica oleracea* seedlings with varying amounts of different nitrogen sources, potassium, and phosphorus. *In situ* movement of the bacteria in infected seedlings was monitored with X-ray film. It was determined that insufficient amounts of nitrogen increased disease, but that amounts above those required for optimum plant growth reduced disease. No significant effects on disease severity were observed from varying the amounts of potassium and phosphorus fertilization; however, when phosphorus was omitted, disease development was greatly inhibited.

The transconjugant was also used to confirm disease transmission via roots and to examine transmission by whiteflies. The bioluminescent pathogen was transmitted through the roots in one out of ten seedlings, which were grown in potting mix containing black rot infected cabbage leaves. The

pathogen was traced from a location in the root system into the leaves with X-ray film. The role of whiteflies in the spread of black rot of cabbage was not determined in this study, because whiteflies did not transmit the pathogen to healthy seedlings.

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Introduction

Advances in technology provide us with new means to study plant disease and make it possible to gain greater insights into the complex relationship between host and pathogen. Until recently, determining the presence, concentration, and spread of bacteria in either asymptomatic or symptomatic plant tissue could be accomplished only by destructive methods, which interrupted the disease process, depended on culture for quantification of the pathogen, and required many replicates. Then, out of the rapidly developing field of genetic engineering, a new tool emerged which was ideally suited to study ongoing plant-microbe interactions in a non-disruptive manner.

In 1986, Shaw and Kado cloned the genes for bacterial bioluminescence into a *Xanthomonas campestris* pv. *campestris* strain and showed how a pathogen could be traced in a cauliflower leaf over a period of days with X-ray film, eliminating the destruction of the leaf during the infection process. The value of a pathogen with light production as a marker for various host-pathogen and epidemiological studies can easily be seen when comparing it to other markers. Antibiotic resistance has been used as a marker for plant pathogenic bacteria, but can pose some problems due to attenuation of the strain (Yuen et al., 1987). Under field or culture conditions, resistance may be lost, or non-target bacteria may express a natural resistance or may readily mutate when placed under antibiotic pressure. Monoclonal antibodies developed for *X. c. campestris* strains are excellent due to their specificity to certain strains (Alvarez et al., 1985; Yuen et al., 1987), but they do not provide the uniqueness that bioluminescence confers upon a single strain to distinguish it from all others. Stably maintained and expressed bioluminescence in a pathogen therefore represents a singular, reliable, safe, relatively inexpensive, and easily used marker.

For this study on black rot of cabbage, the "lux" ("lux" stands for light) genes were transferred from *Escherichia coli* HB101 (pUCD607) (Shaw and Kado, 1986) into a highly virulent *X. c .campestris* strain isolated from a cabbage plant grown on the island of Maui in Hawaii. The transconjugant was thoroughly tested *in vitro* and *in vivo* for stability of expression of the foreign genes and for its competence as a pathogen in comparison to the wild type strain. These tests were a prerequisite to

establish the applicability of bioluminescence as a marker in evaluating the role of host nutrition in the invasiveness of the pathogen, and for planned field release in the future to trace the pathogen in the soil and show possible transmission by whiteflies.

The disease

Black rot of cabbage is a serious disease of world-wide importance (Williams, 1980). The causative organism is *X. c. campestris* (Dye), a Gram negative, rod-shaped bacterium of 0.4 x 1.0 um, with a single polar flagellum. The bacterium is aerobic, catalase and hydrogen sulfide positive, oxidase negative. Nitrate is not reduced and indole is not produced from tryptophan. *X. c. campestris* produces copious amounts of extracellular polysaccharide called xanthan gum. The primary infection sites for the pathogen are the hydathodes, special water pores at the leaf edges from which guttation fluids accumulate as water droplets. Infection can also occur through wounds in all plant parts including roots (Cook, 1952; Williams, 1980). Spread of the pathogen is mainly through infected seeds (Cook et al, 1952; Walker and Tisdale 1920), by splashing water drops, insects (Shelton and Hunter 1985), wind (Kuan et al., 1986), and by mechanical injury. Weed hosts and infested debris in the soil can be sources of inoculum (Schaad and White, 1974; Alvarez and Cho, 1978; Schaad and Dianese, 1981). In the cabbage plant, the bacteria are confined to the xylem vessels (Bretschneider et al., 1989), in which they systemically invade the plant forming typical V-shaped lesions. Chlorosis followed by necrosis develops in the interveinal tissue. The vessel behind the advancing bacterial mass becomes plugged with darkened material which is composed of polysaccharides, melanin-pigmented material, and xylem wall degradation products (Sutton and Williams, 1969; Wallis et al., 1973). This plugging is thought to lead to blackening of the vessels and, water stress. In stems, petioles, and the head vascular blackening can occur without other external symptoms (Cook et al., 1952).

One form of resistance in cabbage to *X. c. campestris* is thought to reside in the hydathodes (Staub and Williams, 1972). In cabbage cultivars, resistance is expressed only after seedlings are six

weeks old or older. This causes concern that young seedlings could become latently infected in the seedbed and would therefore represent a source of inoculum for epidemics after transplant into the field (Williams, 1980; Cook et al., 1952). Head cabbage is the second largest single vegetable commodity produced in Hawaii. In 1989, 16 million pounds, up from 13,300,000 pounds in 1985, were grown on 650 acres, mainly on the island of Maui, with a sales value of $3 million (Hawaii Agricultural Statistics, 1989). The production of head cabbage was previously limited in Hawaii (Alvarez et al., 1987). The practice of continuous cropping combined with favorable environmental conditions promotes build-up of inoculum and spread of the pathogen.

To further study the epidemiology of black rot in Hawaii, *X. c. campestris*tagged with lux could be used to trace the pathogen spread in fields, in the soil, on weed hosts, and potential transmission by insects.

Bioluminescence

Many examples of organisms that emit light exist in nature, e.g., worms, insects, fungi, jellyfish, diatoms, and bacteria. In bacteria, light is produced by oxidation of aldehyde by luciferase. The aldehyde is formed by the reduction of fatty acids by transacetylase and reductase (Hastings et al., 1985). Two reactions summarized below show the need for reduced flavin mononucleotide ($FMNH_2$) , oxygen, and aldehydes to form light (1). The oxidized aldehyde (or fatty acid) is reduced to aldehyde (2). Thus the organism is able to recycle the aldehyde substrate for light production. This is in contrast to the firefly luciferase, which utilizes the cyclic aldehyde luciferin that cannot be recycled.

$$1)\ RCHO + FMNH_2 + O_2 \xrightarrow[\text{(light reaction)}]{\text{luciferase}} RCOOH + FMN + H_2O + hv$$

$$2)\ RCOOH + NADPH_2 + ATP \xrightarrow[\text{(recycling reaction)}]{\text{reductase}} RCHO + NADP + AMP + PPi$$

Techniques in molecular biology have made it possible to identify and clone the genes for bioluminescence. The first report of cloning luciferase genes was by Belas et al. (1982) from *Vibrio harveyi*, a marine bacterium. Engebrecht and coworkers (1984) cloned the luciferase genes as well as the functional fatty acid reductase genes in a single 16 Kb fragment from *Vibrio fischeri*, a marine bacterium which lives in symbiosis with the pine cone fish, *Monocentis japonicus*. The synthesis and level of activity of the enzyme luciferase is regulated by an autoinducer in *Vibrio fischeri* (Hastings and Nealson, 1977). The bioluminescence genes are activated as the concentration of the autoinducer rises with an increase in bacterial numbers. The autoinducer molecule has been identified (Hastings and Nealson, 1977; Nealson and Hastings, 1979; Eberhard et al., 1981). The need for the presence of an autoinducer was eliminated by Engebrecht's research group (1985), who constructed a promoterless "lux" cassette cloned close to the end of a transposable element allowing transcriptional fusions of heterologous promoters to "lux" in *E. coli*. Shaw and Kado (1986) modified the original "lux" cassette, and constructed the broad-host range plasmid pUCD607 in (20.6 Kb), which is able to replicate in many gram-negative bacteria. The "lux" genes are under the constitutive control of a pBR322 tetracycline promoter on this plasmid. The plasmid also confers resistance to ampicillin, kanamycin/gentamycin, spectinomycin/streptomycin, and can be easily transferred into bacteria of interest to create bioluminescent transconjugants that can be monitored during infection of plants.

Bioluminescence is a useful tool in many laboratory analyses. It is being used as a reporter gene in molecular biology (Shaw and Kado, 1987, 1988; Kamoun and Kado, 1990). For a plant pathologist, organisms with the "lux" genes afford non-disruptive disease progression studies (Shaw and Kado, 1986), and a reliable marker in following the spread of the pathogen through the crop, in the seedbed, the soil, into weed hosts, and through insects.

Bioluminescence can be detected and measured in various ways (Shaw and Kado, 1987). The intensity of light production by the bioluminescent bacterium and the nature of the experiment determine which of these methods are most appropriate. Bright colonies can easily be seen with the human eye,

whereas more sensitive instruments are required for "dim" transconjugants. Light production by an organism can be easily recorded with X-ray film or other types of sensitive films (Shaw et al., 1987), and bacterial concentrations in liquid culture can be quantified based on quanta of light emitted by the growing culture.

Host nutrition in relation to disease

The literature is rich in studies on the effects of nutrition of the host plant on pathogenic organisms and the disease process (Colhoun, 1973; Huber, 1985). Numerous studies give strong indication that the nutritional status of the host plant is related to disease severity. A healthy plant, just like any other healthy organism, is thought to be able to withstand infection more easily or become comparatively tolerant to the pathogen. In crop systems, the degree of plant health is usually determined by economics and consumer taste, and may not reflect a "fit" plant with respect to a balanced host-parasite relationship. In this field, research has focused mainly on the control of fungal diseases which are of greater economic importance. A large number of fungal pathogens infect plant roots at the plant-soil interface where all of the nutrients are present. Bacterial diseases have not been as thoroughly investigated, because they represent a relatively small fraction of crop problems. For control of bacterial pathogens on crop plants growers have relied mainly on copper compounds and on the use of antibiotics, but extensive use of antibiotics over a period of time is associated with development of resistance on the part of the pathogen (Moller et al., 1981).

Although many advances in plant physiology have been made, the host's natural defense reactions are not well understood, and no general conclusions can be drawn from the many experiments done on plant nutrition in host-pathogen relationships. Temperature (high, low, fluctuating), light (intensity and daylength), moisture (soil and atmospheric), growth medium and its microbiota, and certain crop practices are all factors to take into consideration in nutrition studies. The physiological and biochemical differences in plant parts which pathogens infect and multiply in must be considered, e.g.,

the growth requirements for a xylem invader may be quite different from those of a phloem invader. Plant nutrient concentrations and ratios can influence plant growth and vigor and its biochemical make-up, thereby providing an environment for the pathogen which can be either favorable or unfavorable (Palti, 1981). Most investigations focus on varying rates of complete fertilizer formulations with an emphasis on nitrogen, phosphorus, and potassium, but the individual effects of these elements have also been examined, and some researchers have tried to establish a role for calcium, magnesium, sulfur, and minor elements in a disease situation (Zoller, 1972; Philip and Devadath, 1984).

Nitrogen is the most intensively studied element in research investigating plant health in relation to disease. Not only is the concentration available to the plant important, but also the specific form, such as nitrate vs. ammonium (Huber and Watson, 1984). High-nitrogen, vigorously growing crops can be attacked by some pathogens, and excess nitrogen can result in a higher degree of disease incidence and severity, but it is believed that the majority of facultative parasites prefers low-vigor crops (Grossmann, 1970, in Palti, 1981).

Fungal vascular wilt symptoms, such as those caused by *Fusarium* in cabbage yellows disease, usually decrease in severity with an increase in nutrient concentrations (Walker and Hooker, 1945; Walker and Foster, 1946; Schroeder and Walker, 1942). Most studies with xylem-inhabiting bacterial pathogens gave similar, but a few gave opposing results. Smith (1944) reported a decrease in infection by *Pseudomonas solanacearum* in tobacco plants at higher amounts of applied nitrogen, and the same pathogen also caused less disease in tomato at high nitrogen levels, but only during longer daylength (Gallegly and Walker, 1949). In a comparative study between two diseases, opposite results were obtained; black rot of cabbage, caused by the xylem pathogen, *X. c. campestris*, became less severe with nutrient increases, but ring rot of tomato, caused by a phloem invader, gave a higher disease index (Walker and Gallegly, 1951). This investigation was done, because previous experimental work on *Corynebacterium michiganense*, which was thought to live in the phloem, but invades cortical tissues

(Pine, 1955), infected tomato plants with increased aggressiveness under increasing nutrient concentrations (Walker and Kendrick, 1948).

The growth of some xylem pathogens can also be enhanced by high fertilization. In an extensive study on bacterial wilt of corn caused by the xylem invader, *Erwinia stewarti* (*Phytomonas stewarti*), investigators found that an increase in the concentration of nitrogen, phosphorus, or potassium beyond the optimum for plant growth increased the severity of the disease (Spencer and McNew, 1938; McNew and Spencer, 1939). The researchers also established that severity of invasion was directly correlated with the total amount of nitrogen supplied the seedlings rather than with the growth of the host. More recent work on anthurium blight, caused by *X. c. dieffenbachia*, determined that high fertilization produced a greater number of leaves with higher susceptibility to the pathogen, and also reduced yield in flower production (Chase, 1989). It was recommended that excessive fertilization be avoided to reduce susceptibility to infection.

Pathogens causing leaf spot and blights also react differentially to varying fertilizer rates, but most seem to respond with a reduction of disease incidence or severity at higher nutrient availability. This was established for the two pathogens responsible for halo blight and common blight of beans, *Pseudomonas phaseolicola* and *Xanthomonas phaseoli*, respectively; increased concentrations of a balanced nutrient solution resulted in suppression of disease development in each of the diseases (Patel and Walker, 1962). Also, on *Philodendron oxycardium* varying concentrations of nitrogen, phosphorus, and potassium were tested, and the researchers concluded that a decrease in disease severity by *Xanthomonas* species could be related to increased N, and that K and P had little effect, although high K stimulated growth without an appreciable increase in disease (Harkness and Marlatt, 1970). In canker of peaches and prunes, *Xanthomonas pruni* produced less severe symptoms on trees grown under high nitrate fertilization (Huber and Watson, 1974). Chase (1984) and Chase and Poole (1987) reported that severity of disease on members of the *Araliaciae* family can be reduced by light and applying high amounts of complete fertilizers. A linear decrease of disease with increasing fertilization rates without a

change in plant growth occurred. These researchers also reported a linear decrease in Ca, Mg, and Na as N, P, and K increased. On *Syngonium podophyllum*, the severity of *Xanthomonas* blight was not only reduced with increased amounts of complete fertilizer, but also by increases of nitrogen or potassium, which were equally effective in reducing symptom expression (Chase, 1989). Chase was able to effectively decrease in a number of diseases caused by *Xanthomonas* pathovars with fertilizer management (Chase, 1988).

Examples to support enhancement of disease from fertilizers are represented by some observations on leaf spot of chrysanthemums; severity of leaf spot caused by *Pseudomonas chicorii* could be correlated to high N and high P in leaves due to high fertilizer rates. It should be noted here, that the same pathogen was inhibited on another host, dwarf schefflera, when the fertilizer rate was increased six times (Jones et al., 1985).

The importance of regarding each host-pathogen system as an entity in nutrition-disease research is supported by results from studies on *Erwinia* blight on *Dieffenbachia* (Chase and Poole, 1982). The greatest degree of resistance to the pathogen was expressed at recommended fertilizer rates which also assured optimum plant health, and at either insufficient or excessive fertilization the number of leaf spots increased.

Host-pathogen interactions inside the plant have not been elucidated, and researchers are trying to explain some of the observations made. In some diseases, high nitrogen was associated with susceptibility (Thomas and Ark, 1936), and it was concluded that the susceptibility could be attributed to a relatively low carbohydrate-high nitrogen content (Nightingale, 1936). Thomas (1965) reported different reactions by two leaf spot pathogens on two varieties of sesami, with one variety showing a higher disease index at both 12- and 16-hr daylength with supplemented nitrogen. The other variety was resistant to both bacteria, but its reaction to supplemented nitrogen was dependent on daylength; it expressed susceptibility to *Pseudomonas sesami* under a 12-hr light regime, but remained resistant to *Xanthomonas sesami* under 16-hr daylength only. Based on previous work by Thomas and Orellana

(1963), these results might be attributed to the differences in the ratio of reducing sugar to amino compounds and utilization of those compounds by the two bacteria. Bird (1954) also thought that resistance to infection on cotton wilt by *Xanthomonas malvacearum* was due to a favorable carbohydrate-nitrogen balance in cotton leaves. He based his observations on the findings that resistant plants contained more carbohydrates than nitrogen in comparison to susceptible plants, and postulated that this could be attributed to the genetic makeup of the plant.

Many researchers attribute the opposing experimental results in various host-pathogen systems to different nitrogen forms utilized by the organisms. Nayudu and Walker (1960, 1961) related composition of total and soluble nitrogen in young and old leaves of tomato to bacterial spot by *Xanthomonas vesicatoria.* Decreased percentages of total and soluble nitrogen in the old leaves correlated with a decrease in lesion severity. Earlier, vanGundy (1957) found that *Pseudomonas lachrymans*, like *X. vesicatoria*, does not use inorganic salts, such as ammonium chloride, ammonium sulfate, ammonium nitrate and sodium nitrate, as a nitrogen source. As amino-N rose in the leaves, disease increased. In the same experiment, low potassium also increased disease, whereas no apparent effect of phosphorus was observed.

Zoller (1966) studied the influence of fertilization on black rot of cabbage in a field experiment. He grew cabbage plants on soils amended with sulfur, ammonium phosphate, and sulfate compounds, and at certain intervals collected guttation fluids from plants to determine generation times of *X. c. campestris* in these fluids. He also infected plants with the black rot pathogen and measured lesion length on plants from different soil treatments. He found that the bacteria were not much affected by the treatments during the first 18 days after soil amendment, but died in guttation fluids from plants grown on sulfur and potassium sulfate by day 21. Lesion size was also reduced on plants from sulfur and sulfate amended soils.

The role of potassium and phosphorus in black rot of cabbage has not been thoroughly investigated, but one study reported no significant effect of these two elements when supplied in

unbalanced solutions (Walker and Gallegly, 1951). Phosphorus by itself has not been implicated as an important factor in black rot, but would most probably become important when not balanced with other elements. One report shows that bean leaves below a critical level of P were susceptible to *Erwinia carotovora*, but expressed HR above that level (Tejerina et al., 1982). *Xanthomonas pruni* produced fewer bacterial spots on plum trees growing with high P than on high-N trees (Bachelder et al., 1956). Phosphorus seemed to have little effect on a *Xanthomonas* disease on an ornamental in general, except in one experiment where a reduction was found when the leaf P exceeded 0.4% (Harkness and Marlatt, 1969). On the other hand, *X. c. pelargonii* became more aggressive at high P concentrations (Kivilaan and Scheffer, 1958).

Studies with potassium on diseases caused by *Xanthomonas* species generally show that high K results in a reduction of disease severity (Bachelder et al., 1956; Matthee and Daines, 1967; Huber and Arny, 1985).

A complex picture of host-pathogen interactions in relation to plant nutrition arises from all of these studies. Environmental factors as well as host and pathogen metabolism play a role, and results from nutrition studies cannot be explained simply as competition between plant and pathogen for the available ions, especially since the mechanism for favored or inhibited growth of the pathogen inside its host is not known.

It is impossible to draw generalizations from the literature which would allow for precise categorization of pathogens and diseases with respect to nutrition of the host. Generally, however, as far as systemically invading xanthomonads are concerned, results from various studies, such as black rot of cabbage and *Xanthomonas* blight of syngonium, show a reduction in disease with higher nitrogen fertilization.

Development of Hypotheses

I. Stability of plasmid pUCD607 genes in Xcc 171LIIH-7. Gene transfer is accomplished by plasmids bearing the gene(s) of interest and one or more antibiotic resistance genes to select for successful transfer. Continued expression of the foreign genes is assured by maintaining the transconjugants on media containing the appropriate antibiotics. However, such transconjugants inside the plant host may quickly lose the hybrid plasmids in the absence of antibiotic selection. Another factor in loss of plasmid DNA is the possibility of a resident plasmid in the recipient which is of the same incompatibility group as the foreign plasmid. It would be desirable to have the genes integrated into the host chromosome or to have a low copy number of the plasmid, such as one or two plasmids per chromosome; those could be stably maintained and inherited (Scherratt, 1982). If the genes are integrated into the chromosome, such an event must not interfere with the regular metabolism of the bacterium. Unanticipated effects, which could not have been predicted from the information on the inserted DNA have been found in phytopathogenic bacteria containing plasmid pRD1 which arose as an unpredicted phenotype (Kozyrovskaya et al, 1984;). The lux plasmid pUCD607, however, contains the stability functions that allow either stability as an independent replicon or by integration by a single recombination event into the chromosome.

In order to gather useful and meaningful information in host-pathogen interactions or other aspects of disease studies, the transconjugant must express the same characteristics as the wild type with respect to growth, virulence, and pathogenicity. In addition, the genes have to be stably expressed under varying experimental conditions.

I propose that the bioluminescent *X. c. campestris* 171LIIH-7 will retain its characteristics as a pathogenic, virulent black rot pathogen in cabbage infections while stably expressing the genes for light production and antibiotic resistances.

II. Host nutrition in relation to disease. To draw conclusions about the nutritional status of the cabbage plant in relation to its degree of susceptibility to the invasiveness of the black rot pathogen

requires the deductive interpretation from available studies with other pathovars of the genus *Xanthomonas* and some other pathogens which infect plants systemically. A close analysis of the many published results points out that each system has to be investigated separately. The two studies done on black rot of cabbage provide good evidence that nutrition has a part in the disease complex that warrants further investigation (Walker and Gallegly, 1951; Zoller, 1966).

With the availability of a bioluminescent *X. c. campestris* I am in the position to look at the disease progression in a more thorough way. Rate and extent of the pathogen's invasiveness into the leaf can be fairly accurately determined without disturbing the disease process, and subtle differences may become known that may not be anticipated visually.

It is my hypothesis that mineral nitrogen compounds applied in different amounts to cabbage seedlings over a period of time prior to inoculation will affect the invasiveness of *X. c. campestris* into the cabbage leaf. I further propose that the pathogen's invasiveness will also be influenced by varying amounts of potassium and phosphorus fertilization of the cabbage seedlings.

CHAPTER II

Introduction

Xanthomonas campestris pv. *campestris* (Xcc) causes black rot of crucifers, which is a bacterial disease of world-wide importance (Williams, 1980). Black rot is serious in regions with tropical climates. Under these conditions, Xcc populations rapidly increase to epidemic proportions promoted by continuous cropping cycles. In the State of Hawaii, 650 acres are in cabbage production, yielding 16 million pounds of cabbage with a market value of $3 million annually (Hawaii Agricultural Statistics Service Branch. Hawaii State Dept. of Agriculture, 1989).

Since it was first described by Russell (1898), the disease has been studied extensively, but without much understanding of the infection and disease process. Gene technologies are expected to reveal a much deeper understanding of the mechanisms underlying host-pathogen interactions.

Epidemiological studies on black rot in the greenhouse or in the field should ideally be done with reliable markers on the pathogen to trace its spread by various means eliminating the possibility of interference of other strains which could already be present in the environment or are entering the test site. Markers are also required in mixed culture or comparative studies of closely related strains. Antibiotic resistance as a marker is not reliable, because antibiotic resistant mutants of *X. c. campestris* may lose resistance under field conditions in the absence of antibiotic pressure. In addition, since some mutants are attenuated strains, they may exhibit other differences from the wild type (Yuen et al., 1987). Monoclonal antibodies are excellent markers due to their specificity (Alvarez et al., 1985; Yuen et al., 1987; Benedict et al., 1989), but cannot distinguish among strains with the same antibody reaction profile. A more appropriate marker is therefore needed.

The study of host-pathogen interactions on or in the plant, particularly growth rates of the pathogen, inhibition or stimulation, and invasiveness require a large number of replicates to compensate

for the genetic variability of each plant to obtain a meaningful statistical evaluation. Furthermore, monitoring the disease process in the plant over a period of time has been a disruptive and destructive process in addition to requiring much work input.

Non-disruptive disease progression studies have been made possible by the isolation of a luciferase operon, "lux", from the marine bacterium *Vibrio fischeri* (Engebrecht et al., 1984). Shaw and Kado (1986) cloned the genes into a broad-host range plasmid and put the "lux" genes under the constitutive control of a pBR322 tetracycline promoter to generate vector pUD607. The plasmid was transferred by conjugation from *E. coli* HB101(pUCD607) to *X. c. campestris* 2D520, and stable bioluminescence in this bacterium was assured by integration of the plasmid genes into the bacterial chromosome. These researchers were then able to record the progression of the bioluminescent bacterium in a cauliflower leaf with X-ray film without disrupting the infection. The value of bioluminescence as a marker lies in its reliability, simplicity of identification, safety of handling, and relatively inexpensive application. Brightly glowing bacteria can easily be seen in the dark on appropriate media, and growing inside the host plant.

In this study I describe basic experiments to isolate a suitable bioluminescent *X. c. campestris* for our studies on black rot of cabbage in Hawaii. Valid conclusions about experimental results from studies with a bioluminescent Xcc can only by arrived at if the transconjugant behaves identically to the wild type in the various aspects of the disease process under investigation. Therefore, a suitable transconjugant has to be as pathogenic and virulent as its wild type, and must stably express bioluminescence *in vitro* and *in vivo*. Furthermore, before a bioengineered organism can be safely released into the natural environment, it is imperative to establish that it does not pose a threat to plants or animals or beneficial microorganisms. Integration of the plasmid-encoded genes into the bacterial chromosome would lower the risk of gene transfer to other organisms.

Materials and Methods

Bacterial strains and plasmids used are described in Table 1. *X. c. campestris* G-171 is a highly virulent black rot pathogen on cabbage and other crucifers tested (cauliflower, radish, broccoli, mustard weed), which readily infects crucifers through the hydathodes when spray-inoculated. Strain G-171 is naturally resistant to ampicillin. Chloramphenicol (Cm) was used as the resistance marker for the isolation of a suitable bacterium for transformation. A culture of G-171 was grown on medium 523 (Kado et al., 1972) for two days, diluted to $A_{600} = 0.1$, and spread on 523 agar plates containing 25 ug/ml Cm, and a Cm resistant mutant colony was spontaneously generated on successively higher concentrations of the antibiotic. A chloramphenicol resistant *X. c. armoracia* strain 756 mutant and a rifampicin-resistant *X. c. campestris* strain 84-81 mutant were also generated.

Transfer of plasmid pUCD607 into G-171 Cm^r, 756 Cm^r, and 84-81 Rm^r. Plasmid pUCD607 containing the "lux" gene cassette is maintained in *E. coli* HB101 (Shaw and Kado, 1986). The plasmid was transferred into the Cm and Rm mutants in a triparental mating using *E. coli* pRK2013 (Figurski and Helinski, 1979) as the mobilizing strain. The donor and helper strains were grown on Luria plates (Miller, 1972) with the appropriate antibiotics at 37 C; the recipient strains were grown at 28 C on 523 agar in the presence of appropriate antibiotics. The mating mixture consisted of a loopful of a two-day old culture of the recipient and an overnight culture of the donor and helper, which were placed in 1 ml NYGB (g/l: peptone 5, yeast 3, glycerol 20), spun in a microcentrifuge for 15 seconds and washed twice in NYGB. The final pellet was resuspended in 100 ul of NYGB and pipetted directly on a NYG agar plate or on a 45 u Millipore filter placed on a NYG agar plate, and incubated for 24 hours at 30 C. The mating mixture was then spread on selective 523 agar plates containing 40 ug/ml Sp and 50 ug/ml Cm for the Cm resistant mutants, and 100 ug/ml Rm for the Rm resistant mutant. The plates were incubated at 28 C until colonies appeared, then moved to 25 C to monitor appearance of bioluminescent colonies. Twelve light-producing colonies of G-171, eleven of 756, and 9 of 84-81 were streaked for purity on 523 agar plates containing the required antibiotics.

Table 1. Bacterial strains and plasmids.

Strains or Plasmids	Relevant characteristics	Source or Reference
Strains		
***E. coli* HB101**		H. Kamdar
X. *campestris pv. campestris*		
G-171	wild type isolate from cabbage; Ap^r	A. Alvarez
171CM	spontaneous chloramphenicol resistant mutant of G-171	this study
171LIIH-7	Cm^r Ap^r; 171CM containing pUCD607	this study
171LIIH-6	same as 171LIIH-7	this study
84-81	Wild-type isolate from wild mustard	R. Campbell (U.C.D)
84-81RF	Spontaneous Rm^r mutant of 84-81	this study
84-81RUH	Rm^r; 84-81RF containing pUCD607	this study
2D520(pUCD607)	Rm resistant mutant of 84-81RF containing pUCD607	Shaw and Kado (86)
X.*campestris* pv. *armoraciae*		
756	wild type isolate from cabbage	California
756CM	spontaneous chloramphenicol resistant mutant of 756	this study
756LIIH	Cm^r ; 756CM containing pUCD607	this study
Plasmids		
pUCD607	tet-lux fusion; oripSa oripBR322 Ap^r Km^r (Gm^r) Sp^r(Sr^r)	Shaw and Kado(86)
pRK2013	Tra^+ Mob^+ Km^r; COLEI replicon	Figurski & Helinski (1979)

Agarose gel electrophoresis. Plasmid DNA was extracted from the transconjugants via the miniprep alkaline lysis method (Birnboim and Doly, 1979), and presence or absence of plasmid confirmed by electrophoresis on a 0.4% agarose gel at 80V, stained with ethidium bromide, and photographed under ultraviolet light (300 nm).

Serial passage on agar medium. Stability of expression of the plasmid-borne genes in the transconjugants during subculturing was tested by serially passing selected cultures four times on 523 agar supplemented with antibiotics. The first culture was streaked from the original isolate stored at -80 C in 523 and dimethylsulfoxide (0.7 %). Bacteria were grown at 28 C and transferred to fresh medium every three days.

Pathogenicity tests on cabbage seedlings. *Brassica oleracea* cv. *capitata*, G-C cross, seedlings (seeds obtained from Dr. Cho, Dept. of Plant Pathology, University of Hawaii) were grown in "Supersoil Potting Mix" (Rod McLellan Co.) in the greenhouse for six weeks and spray-inoculated with the transconjugants at an inoculum concentration of approximately 10^8 cfu ml^{-1}. Control plants were inoculated with strains G-171, 756, and 84-81. One set of seedlings was also wound-inoculated with strain 84-81. Pathogens were reisolated from developing lesions and tested for bioluminescence and plasmid-borne antibiotic resistance on agar plates. Two transconjugants, 171LIIH-7 and 171LIIH-6, were selected for serial passages through cabbage plants to determine whether pathogenicity, virulence, and bioluminescence remained unaffected by the foreign DNA.

Transformation of *E. coli*, and CsCl gradient centrifugation. Transformation of *E. coli* DH1 competent cells, and gradient centrifugation were employed to determine if free plasmid DNA resided in the transconjugants 171LIIH-6 and 171LIIH-7. DNA was extracted by the alkaline lysis method (Birnboim and Doly, 1979). For the transformation, the entire extract from a saturated 5 ml culture was mixed with 100 ul of *E. coli* competent cells, placed on ice for 20 minutes, followed by heat shock for 5 minutes at 37 C. The bacteria were grown in 1 ml Luria broth for 1 hour on a rotary shaker, plated on Luria agar containing ampicillin, and incubated at 37 C. As soon as colonies appeared, the agar plates

were moved to room temperature to detect bioluminescent colonies. Controls consisted of *E. coli* cells transformed with purified pUCD607 DNA. For the gradient centrifugation, DNA was extracted from a 500 ml culture grown to saturation.

Southern hybridization for location of plasmid genes in 171LIIH-7. To determine whether or not pUCD607 had integrated into the chromosomal DNA of 171LIIH-7 and 171LIIH-6 strains, Southern hybridization using pUCD607 DNA as a probe was used. Plasmid DNA was extracted from *E. coli* (pUCD607) by the alkaline lysis method (Birnboim and Doly, 1979) and purified by equilibrium centrifugation in a CsCl density gradient. Miniprep total genomic DNA was isolated from G-171, 171LIIH-7, 171LIIH-7PR (reisolate from plant infection), 171LIIH-6 and 2D520(pUCD607) (Shaw and Kado 1986) by the CTAB method (Current Protocols in Molecular Biology, 1987). Both plasmid and genomic DNA was digested with 1) restriction enzymes (Boehringer Mannheim) Asp718 and SalI, which cut the 20.6 Kb plasmid into three fragments, and 2) Eco RI and SalI, which cut the plasmid into 4 fragments. The resulting DNA fragments were separated by electrophoresis in a 0.7 % agarose gel and transferred to a Zeta-nylon membrane (Biorad) by Southern hybridization protocol (Biorad Bulletin 1234). The membrane was probed with ^{32}P labeled pUCD607 DNA by random priming (Random Primed DNA Labeling Kit, Boehringer Mannheim).

Growth in liquid medium. Growth of G-171 and 171LIIH-7 separately and in mixed culture was determined as follows. Inoculum was prepared by adjusting overnight cultures to $A_{600} = 0.1$ and further diluting by 1000 (calculated inoculum concentration being equal to approximately 10^6 cfu ml^{-1}). One milliliter of this dilution was inoculated into 30 ml of 523 broth (calculated inoculum concentration being equal to approximately . For the mixed culture, 0.5 ml from each diluted culture was used for inoculation. The cultures were grown at 27 C on a shaker starting at 160 rpm and increasing to 200 rpm as the bacteria reached a greater concentration. At 0, 2, and 4 hours and every four hours thereafter up to 32 or 36 hours, 1 ml samples were removed and diluted for colony counts (five 20 ul spots per dilution) on 523 agar plates. For the mixed culture, dilutions were pipetted on both 523 agar plates and on 523

containing 50 ug ml^{-1} spectinomycin. The number of colony forming units (cfu) for the wild type was calculated by subtracting the number of transconjugant colonies on Sp medium from the total number of colonies on 523 agar. This procedure was used, since bioluminescent colonies cannot be accurately counted in the dark.

Growth at different temperatures. G-171 and 171LIIH-7 were grown in 20 ml of 523 broth at 180 rpm on a shaker at 25, 28, 32, and 34 degrees. Inoculum was prepared to give an initial cell concentration of approximately 10^6 cfu ml^{-1}. Bacterial growth was recorded using a Klett densitometer at 590-640 nm. A standard curve for Klett readings vs. bacterial numbers was prepared at 28 C.

The effect of temperature on bioluminescence. A suspension of 171LIIH-7 was spotted in 5 ul spots on 523 agar plates and grown at 25 C for 48 hours. Bioluminescence was recorded with X-ray film (Fuji Medical) in a 20-second exposure. One plate each was then incubated at 25, 28, 30, 32, and 34 C for 2 hours, after which time bioluminescence was again measured with X-ray film for 20 seconds. All plates were reincubated at 25 C for a further two hours and the exposure repeated. 171LIIH-7 was also tested for light production at 16 C.

Monoclonal antibody reactions. Bacterial isolates G-171, 171LIIH-7, 171LIIH-6, 756, 84-81, and 2D520(pUCD607) were tested against a panel of monoclonal antibodies, some specific for *Xanthomonas* species (X1, X11), some specific for Xcc (X9, X13, X17, X21), and others (X16) for identifying and grouping *X. c. campestris* strains (Alvarez et al., 1985; Yuen et al., 1985). This was done to confirm that the known reactive antigenic sites were conserved in the transconjugant.

Results

Stability of plasmid gene expression in Xcc 171LIIH, 756LIIH, and 84-81LRUH strains. Except for two 171 transformants, 171LIIH-6 and 171LIIH-7, the presence of pUCD607 in the transconjugants was confirmed by agarose gel electrophoresis for all isolates. No plasmid bands could be detected for these transformants, even when concentrated amounts of DNA from minipreps were

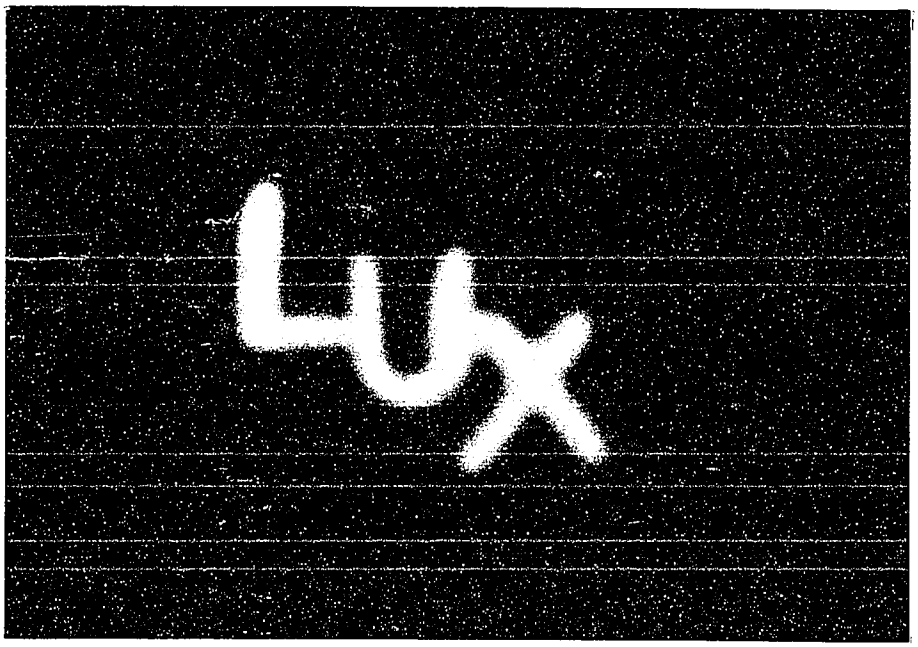


Fig. 1. Bioluminescence produced by the transconjugant *Xanthomonas campestris* pv. *campestris* 171LIIH-7 growing on an agar plate.

examined (Fig. 2). Therefore, pUCD607 likely integrated into these strains. During serial passage on agar medium, all transconjugants continued to bioluminesce on both kanamycin and spectinomycin medium.

Pathogenicity tests on cabbage seedlings. All 171LIIH transconjugants produced typical black rot lesions which were indistinguishable in severity and rate of development from those usually observed in infections caused by the wild type (Fig. 3, A and B). The 756LIIH transconjugants caused typical leaf spots comparable to 756 wild type infections. The 84-81LRUH transconjugants and Xcc 84-81 (wild type) did not produce black rot. Occasional short black veins of not more than 5-10 mm were observed on wound-inoculated seedlings only, but failed to develop into infections, while spray-inoculated seedlings had a few tiny spots which disappeared within a few days. Bacteria could be reisolated from the short black veins only. With the exception of 171LIIH-6 and 171LIIH-7, none of the reisolated transconjugants expressed plasmid-encoded antibiotic resistances, nor the ability to bioluminesce (Table 2). However, in 171LIIH-6 and 171LIIH-7 the plasmid genes were stably expressed in further six serial passages through cabbage seedlings without any loss in pathogenicity, virulence, antibiotic resistance, and bioluminescence expression.

The ability of 171LIIH-7 to strongly bioluminesce while growing in a cabbage leaf was confirmed by a leaf blot taken with X-ray film (Fig. 3B). This transconjugant was chosen for further studies on black rot of cabbage based on its brightness of light production. Strain 171LIIH-6 bioluminesces very dimly and therefore is not suitable for my purposes.

Transformation of *E. coli*, and CsCl gradient centrifugation. *E. coli* competent cells failed to transform with DNA extracted from 171LIIH-6 and 171LIIH7, whereas the control, purified pUCD607 DNA, transformed cells, as confirmed by bioluminescent *E. coli* colonies. In addition, no plasmid DNA band could be detected in the CsCl gradient. These results indicate that the plasmid is not present as an extrachromosomal piece.

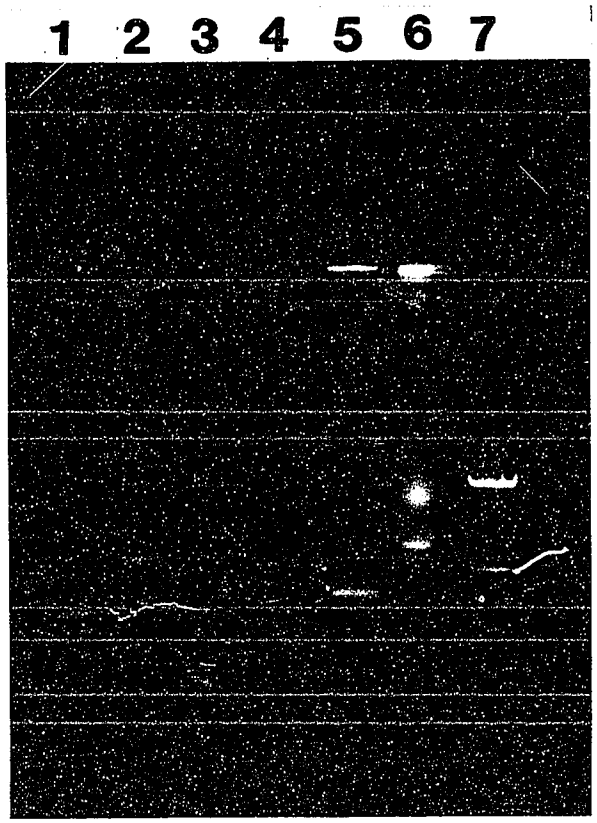
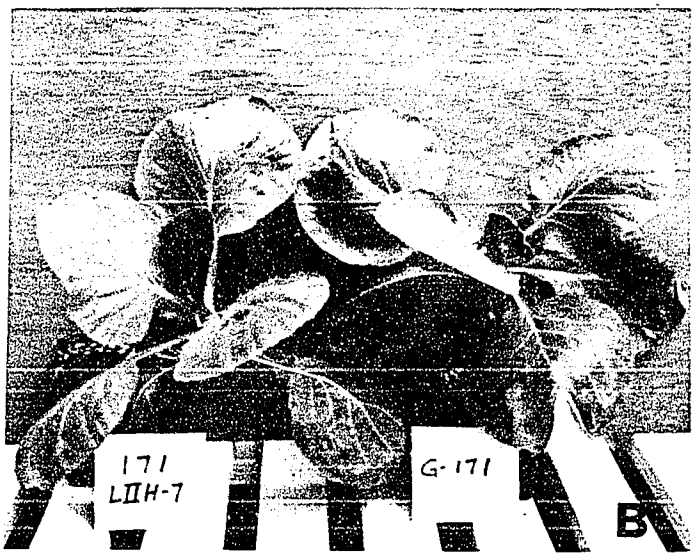


Fig. 2. Agarose gel electrophoresis of plasmid DNA extracted from various bioluminescent *Xanthomonas* transformants. Lanes 1 - 5: transconjugants 171LIIH-4, 171LIIH-7, 171LIIH-6, 171LIIH-8 and 171LIIH-9; lane 6: *E. coli* (pUCD607); lane 7: digested lambda HindIII standard. The absence of plasmid bands in lanes 2 and 3 suggests integration of the plasmid into the chromosome.

Fig. 3. Black rot infection in cabbage seedlings produced by transconjugant *X. c. campestris*171LIIH-7. A) Black rot lesions on four-week old seedlings spray-inoculated with strain 171LIIH-7 at a concentration of approximately 10^8 cfu ml^{-1}. B) Comparison of black rot lesions on seedlings infected with G -171 and 171LIIH-7. C) X-ray exposure (leaf blot) of a cabbage leaf infected with strain 171LIIH-7.



C

TABLE 2 . Growth and bioluminescence of bacteria reisolated from infected cabbage seedlings

	Growth on Medium		
Strain	**523**	**523 Km_{25}**	**Bioluminescence**
171 LIIH-5	+	-	-
-6	+	+	+
-7	+	+	+
-11	+	-	-
756 LIIH-2 *	+	-	-
-4	+	-	-
-7	+	-	-
-11	+	-	-
84-81 LRUH-2 **	+	-	-
-6	+	-	-
-7	+	-	-
-8	+	-	-

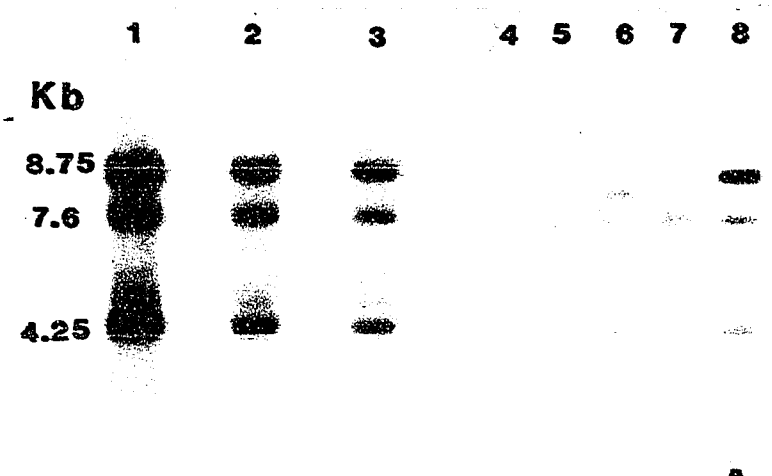
* x.c. pv. armoraciae (leaf spot bacterium)

** Xcc: not pathogenic on cabbage

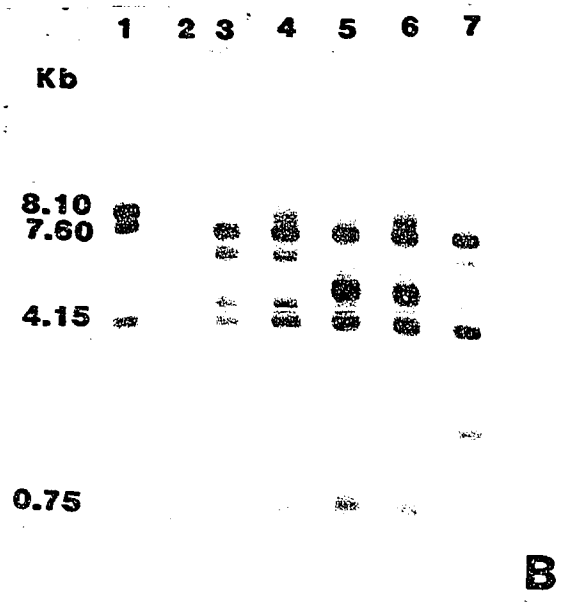
Southern hybridization. The patterns from hybrizidation of the labeled plasmid DNA to digested plasmid and total genomic DNA are shown in Fig. 4. The three expected fragments of plasmid DNA digested with Asp718 and SalI are visible. The 7.6 Kb "lux" band and the 4.25 Kb band are conserved for all transconjugants tested. Hybridization patterns for 171LIIH-7 and 171LIIH-7PR isolates are identical, but the 8.75 Kb is replaced by a piece between 7.6 and 8.75 Kb, and there is a new piece smaller than 4.25 Kb (Lanes 5 and 6). In 171LIIH-6, the 8.75 Kb band is also missing, but a much larger band between 9 and 23 Kb can be seen (Lane 7). The DNA of strain 2D520 has a pattern identical to the digested plasmid. In Fig. 4 B, the DNA of 171LIIH-6 and 171LIIH-7 should hybridize to the probe in the same pattern if pUCD607 was an extrachromosomal element. However, the patterns are different; three of the four expected fragments are conserved (lanes 3 and 5), including the 7.6 Kb piece (the "lux" cassette), but the 8.1 Kb piece is missing. Two new hybridization bands (6.5 and 4.6 Kb) appear for 171LIIH-7, and one (5.3 Kb, a possible doublet) for 171LIIH-6. When plasmid DNA was added to chromosomal DNA (lanes 4 and 6), the 8.1 Kb piece hybridized. These results suggest that the plasmid opened at a site located within the 8.1 Kb fragment and integrated into the chromosome.

Growth of 171LIIH-7 in vitro compared to the wild type. The bacterial growth curves (Fig. 5) show that in liquid culture, the transconjugant grows at the same rate as the wild type. In mixed culture, 171LIIH-7 does not lag behind G-171 in spite of the high energy requirement for bioluminescence (Hastings, 1978). A longer lag phase occurred for the transconjugant at 32 C and 34 C, which can be considered inhibitive to the transconjugant. No inhibitory temperature effect occurred between 28 C and 34 C when bacteria grew on agar plates, but both the wild type and the transconjugant grow more slowly at 25 C.

Fig. 4. Southern hybridization. 1 ug of CsCl purified pUCD607 DNA and 10 ug of miniprep extracted genomic DNA were digested with different restriction enzymes, and electrophoresed on a 0.7% agarose gel, transferred to nylon membrane and hybridized at 65 C with ^{32}P-labeled pUCD607. A) DNA digested with Asp718 and SalI. Lanes 1 - 3: 10x, 20x, and 50x dilutions of pUCD607. Lanes 4 - 8: G-171 (wild type), 171LIIH-7, 171LIIH-7PR (transconjugant after serial passage through cabbage seedlings), 171LIIH-6, and 2D520. B) DNA digested with EcoRI and SalI. Lane 1: 2x dilution of pUCD607 DNA. Lanes 2 - 7: G-171 (wild type), 171LIIH-7,171LIIH-7 and 5 ng pUCD607, 171LIIH-6, 171LIIH-6 and 5 ng pUCD607, and 2D520.



A



B

Fig 5. Growth curves of *X. c. campestris* strains G-171 (wild type) and 171LIIH-7 (transconjugant). Bacteria were grown in 523 broth at 28 C on a rotary shaker as separate and mixed cultures.

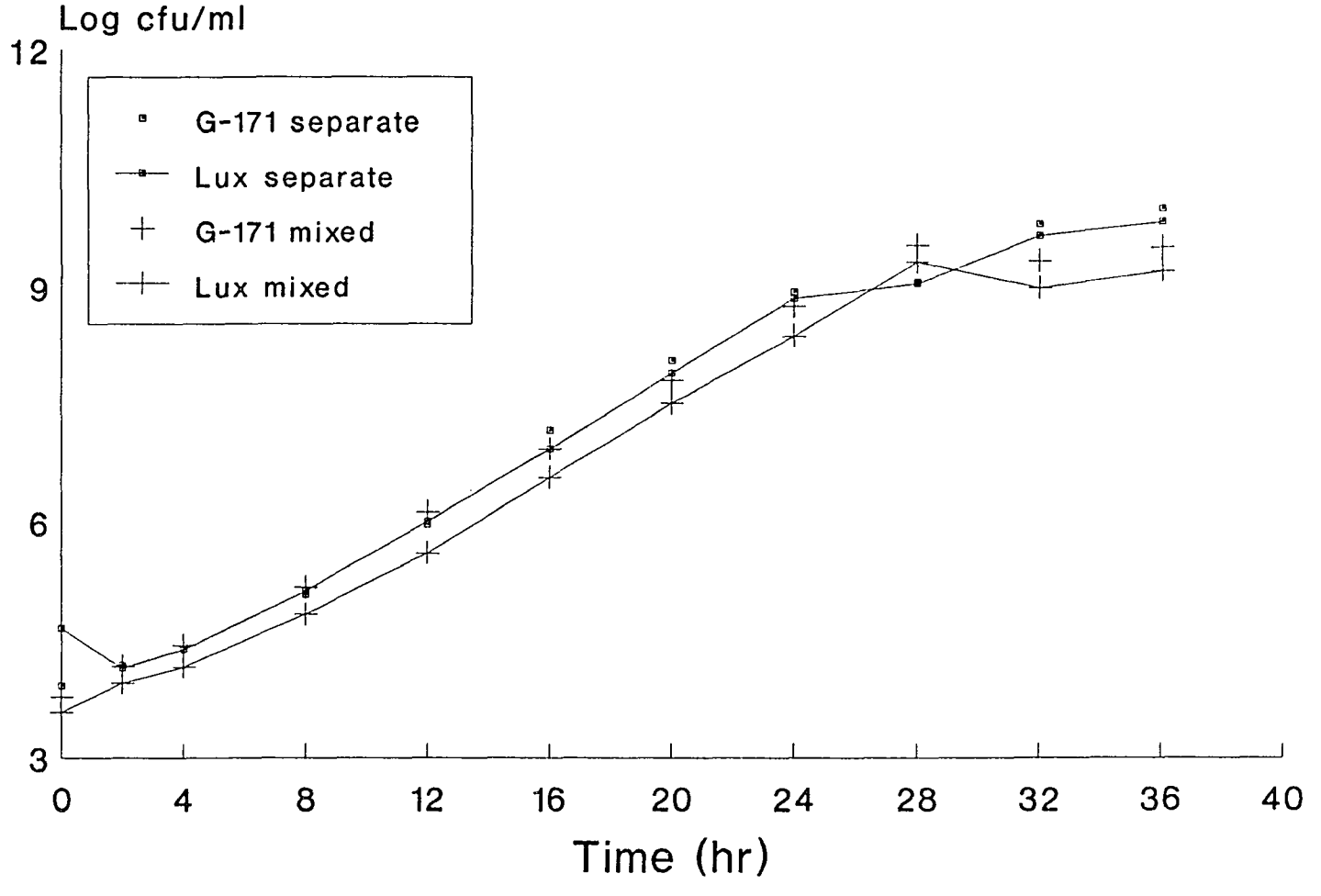
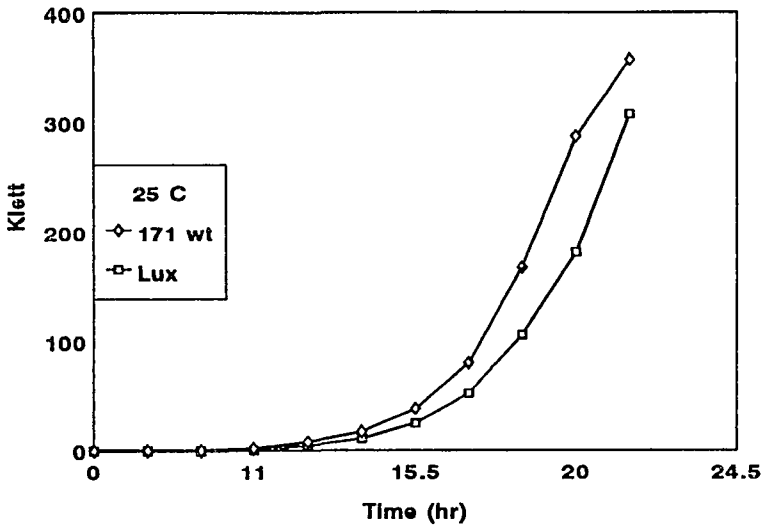
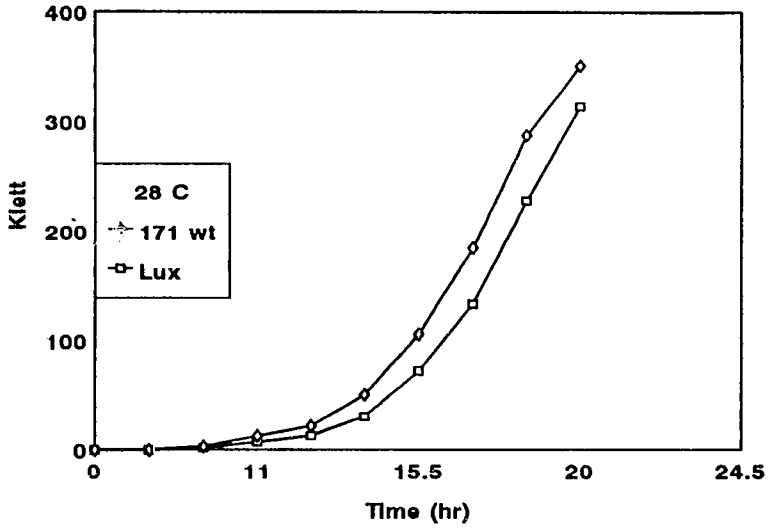
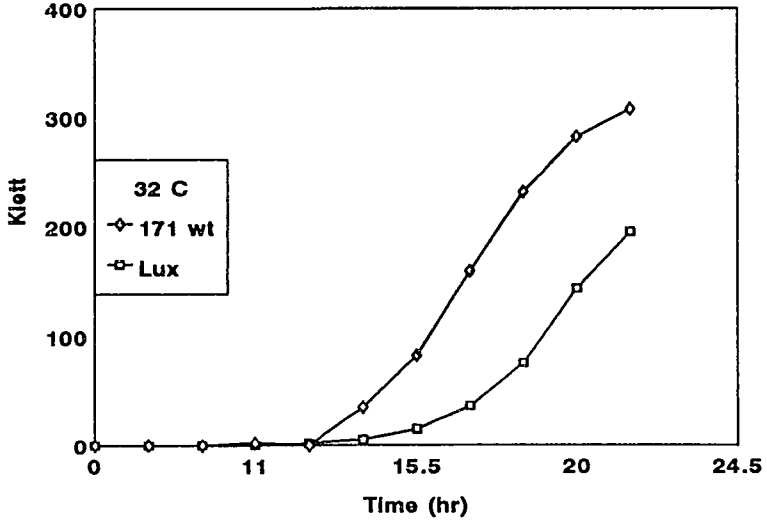
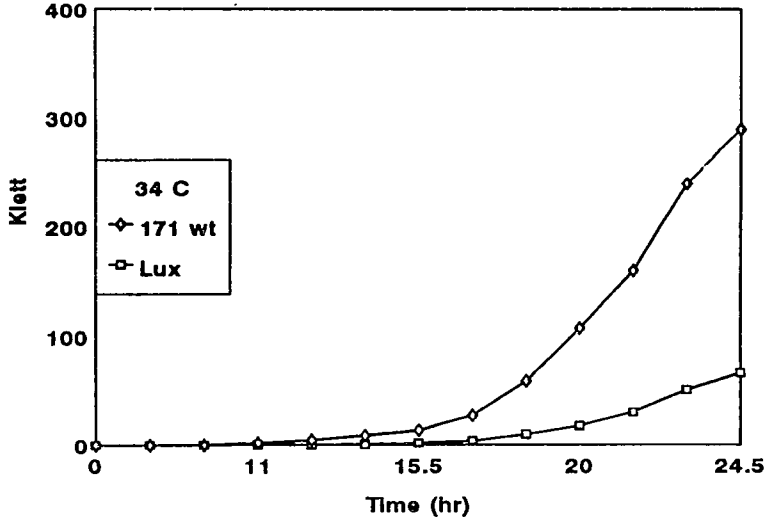

Log cfu/ml
12
9
6
3
G-171 separate
Lux separate
G-171 mixed
Lux mixed
0
4
8
12
16
20
24
28
32
36
40
Time (hr)

Fig. 6. Growth curves of *X. c. campestris* strains G-171 (wild type) and 171LIIH-7 (transconjugant) at different temperatures. Bacteria were grown in 523 broth at 28 C on a rotary shaker.


400
300
200
100
0
Klett
25 C
171 wt
Lux
0
11
15.5
20
24.5
Time (hr)


400
300
200
100
0
Klett
28 C
171 wt
Lux
0
11
15.5
20
24.5
Time (hr)


400
300
200
100
0
Klett
32 C
171 wt
Lux
0
11
15.5
20
24.5
Time (hr)


400
300
200
100
0
Klett
34 C
171 wt
Lux
0
11
15.5
20
24.5
Time (hr)

Temperature-dependent bioluminescence. The temperature effect on bioluminescence in 171LIIH-7 is demonstrated in Fig. 7. The transconjugant is brightest (visually determined) at 25 C. Bioluminescence begins to dim at 30 C, and is absent at 32 C and 34 C for the period of exposure (Fig. 7, lane 2). When the cultures are removed from the higher temperatures and reincubated at 25 C, bioluminescence is reactivated (Fig. 7, lane 3). It was determined that 171LIIH-7 also produces light at 16 C with the same intensity as at 25 C (results not shown). No light measurements were taken from liquid growth cultures due to the unavailability of sensitive instruments.

Monoclonal antibody reactions. The reaction for each strain is shown in Table 3. The insertion of foreign genes did not alter the surface antigens on the transconjugants, 171LIIH-6 and 171LIIH-7. The strains 84-81 and 2D520(pUCD607) show a different reaction pattern from the *X. c. campestris* strains 171; their reaction with monoclonal antibodies X13, X20, and X21 was negative as opposed to positive for 171 strains, but they reacted positively with X16, which does not react with 171 strains.

Discussion

Bioluminescence in a black rot pathogen is ideally suited as an easily handled, inexpensive marker in tracing the spread of bacteria in epidemiological studies, and in progression in the plant during host-pathogen interactions. The bioluminescent *X. c. campestris* 2D520(pUCD607) does not infect cabbage as shown by the failure to produce black rot symptoms in pathogenicity tests for *X. c. campestris*. The ability of the bacterium to infect cabbage leaves through the hydathodes was negative; so was virulence, which is characterized by the multiplication rate of the pathogen in the host and the degree by which it causes typical V-shaped lesions in cabbage leaves. Weakly virulent pathogens invade, cause localized blackening of tissues, but do not spread, whereas highly virulent pathogens spread systemically and cause V-shaped lesions within 7-10 days.

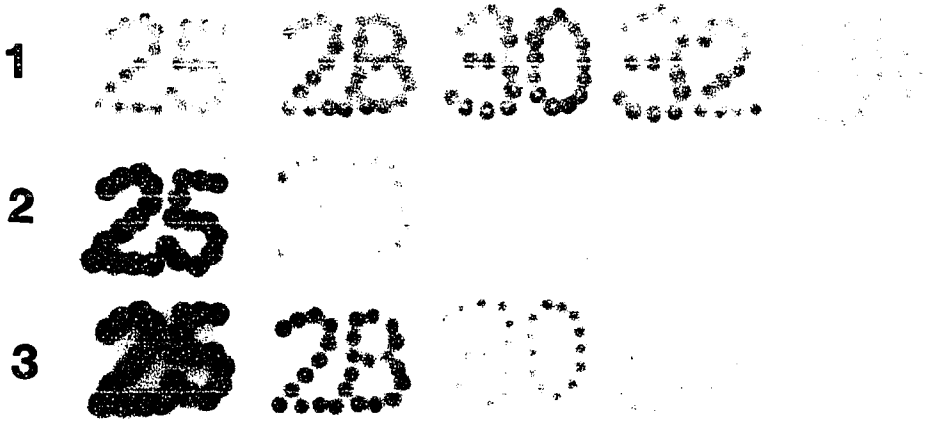


Fig. 7. Temperature effect on bioluminescence of transconjugant 171LIIH-7. Lane 1: X-ray photographs of light production by bacteria two days after incubation at 25 C; lane 2: X-ray photographs after incubation for two hours at the indicated temperatures; lane 3: X-ray photographs after reincubation for two hours at 25 C. Film was exposed to bioluminescent cultures for 20 seconds.

Table 3.Monoclonal antibody reactions of *Xanthomonas* strains

	Monoclonal antibodies							
Strain	X1	X9	X11	X13	X16	X17	X20	X21
G-171	+	-	+	+	-	+	+	+
171LIIH-7	+	-	+	+	-	+	+	+
171LIIH-6	+	-	+	+	-	+	+	+
84-81	+	-	+	-	+	+	-	+/-
2D520 (pUCD607)	+	-	+	-	+	+	-	+/-

The monoclonal antibody reactions suggest that strains 84-81 and 2D520(pUCD607) have a very low degree of virulence. Mabs X9, X13, X21, and X17 react with 98% of the virulent strains of *X. c. campestris*. A positive reaction with two or more of these Mabs is generally indicative of a typical virulent black rot organism. If only one of the four Mabs reacts, the strain is generally less virulent or produces atypical symptoms that develop slowly (Alvarez, 1987). Strains G-171, 171LIIH-6, and 171LIIH-7 fall into the "virulent strain" category. Strains 84-81 and 2D520(pUCD607), on the other hand fall into the "weakly virulent" category. They reacted positively only with X17, very weakly with X21, and negatively with X9 and X13. Although monoclonal antibody reactions suggested a low degree of virulence for strain 2D520(pUCD607), based on the above described grouping, this strain does not infect cabbage at all even when the plants are wound-inoculated.

My work on black rot of cabbage and planned additional projects require a pathogen highly virulent on this host, which can stably maintain and express the bioluminescence genes. For this reason I isolated a highly virulent transconjugant appropriate for my research. Transconjugant 171LIIH-7 meets the prerequisites for disease progress studies in the greenhouse and epidemiological studies in the field. This transconjugant has been stable in storage at -80 C for three years and through several plate culture transfers. It has been reported that free-living bioluminescent bacteria become non-luminous in culture (Keynan et al, 1961; Nealson, 1972).

In 171LIIH-7, plasmid-borne resistances as well as natural and induced resistances are expressed under laboratory culture conditions and are retained after growth in cabbage plants. Bioluminescence has been reliable. The transconjugant displays identical characteristics to the wild type under all experimental conditions, except for a sensitivity to higher temperatures in liquid culture. On agar plates, however, this temperature sensitivity did not occur. The reason for this difference in temperature response is not clear, but might be explained by differential amounts of extracellular polysaccharide being produced by the two bacteria. I have noticed that cultures of 171LIIH-7 are not as turbid as the

wild type cultures having the same viable count. This corresponds to observations on agar cultures where the wild type is slightly more mucoid. Extracellular polysaccharide probably affords a certain amount of protection to the bacteria, but in liquid culture the cells are suspended and moving around, and are consequently more exposed than on an agar plate. Another possibility exists: the presumed integration of the plasmid into the chromosome happened at a site involved in temperature-related processes; yet, 171LIIH-6, which shows a different chromosomal integration pattern, is also temperature sensitive.

The results from the growth curves (Fig. 5) showed that the transconjugant grows at the same rate as the wild type. Considering the added gene load in the transconjugant, this was surprising when the two bacteria were grown in mixed culture, because the chemistry of bacterial bioluminescence is a high-energy consuming process. Light emission requires an energy consumption of 6 ATP for each photon released (Hastings, 1978), and luciferase accounts for as much as 5% of the soluble cell protein in *Vibrio fischeri,* in which the genes are regulated by the cell metabolism. In 171LIIH-7, the genes for bioluminescence are constitutively expressed *in vitro* and *in plantae* without an apparent self-limiting system. A pathogen with foreign genes conferring such high energy needs on the metabolism might be expected to be either less fit, or conversely become more aggressive in the plant in order to compensate for the additional energy needs. The expression of foreign genes in 171LIIH-7 has not resulted in any obvious reduction or increase in the virulence of the pathogen, and seems to fulfill the requirements for a suitable traceable pathogen for further studies of black rot of cabbage.

However, before a bioengineered organism can be responsibly released into the environment certain precautions have to be taken into consideration. One of these is to evaluate the possibility of gene transfer to other organisms. A list of attributes of organisms and environment for possible consideration in risk evaluation was given by the Ecological Society of America (Tiedje et al, 1989). The genetically engineered organism must not only be fit and possess gene stability, it also must not pose any threat of contamination of other organisms in the environment. Transconjugant bacteria may exchange genetic

material with other strains through conjugation, transduction, or transformation (Lenski, 1987; Miller, 1988). Genetic transfer of medically important bacterial is well documented (Herriott et al., 1970, Wise et al., 1973), but transfer efficiency is low in naturally competent cells (Hamilton et al., 1981). Direct evidence for gene transfer between plant pathogenic bacteria has been reported from *E. herbicola* and *P. syringae* donors to *E. amylovora* recipients (Lacy et al., 1984) and between *Xanthomonas* and *Erwinia* species (Manceau et al., 1986), and plays a major role in conferring resistance to pathogens under antibiotic or heavy metal pressure (Kerr, 1965 and 1969; Lai et al., 1977; Stall et al., 1986; Bender and Cooksey, 1986; Burr et al., 1987). The potential for gene exchange exists continually in the environment, and heavy antibiotic use in the world's agricultural systems poses a threat to animals and humans through the spread of resistance genes in the bacterial world. Some broad-host range plasmids, RP4 to name one, are very promiscuous (Liu et al, 1984). It has also been reported that cryptic plasmids may be able to mobilize other plasmids (Staskawicz et al., 1981). Since 171LIIH-7 carries a number of antibiotic resistances on the plasmid, it is important to consider the possibility of gene transfer to other bacteria.

I believe that the broad-host range plasmid pUCD607 is integrated into the chromosome of 171LIIH-7. Attempts to reisolate plasmid DNA from the transconjugant failed, competent *E. coli* cells were not transformed with concentrated miniprep plasmid DNA from 171LIIH-7, and back-matings into *E. coli* and other *Xanthomonas* strains failed. No plasmid bands were ever detected in agarose gel electrophoresis, even when minipreps were concentrated, and the Southern hybridization autoradiogram banding pattern suggests integration. This is very desirable; integration assures stability to a certain extent and eliminates the possibility of random intrageneric and intergeneric DNA transfer. Furthermore, if 171LIIH-7 could donate plasmid pUCD607 to other *Xanthomonas* species, its uniqueness would be lost.

Hybrid plasmids are often lost during growth of the pathogen in the plant (Shaw and Kado, 1986). My experimental results lead me to the conclusion that stable maintenance of the plasmid-borne genes in all *Xanthomonas* transconjugants tested (these include 21 bioluminescent *X. c. dieffenbachia*,

unpublished data) depends on integration into the chromosome or very low copy number, since only two out of forty-one transconjugants retained the genes during growth in their host. These two are 171LIIH-6 and 171LIIH-7, both of which seem to have the plasmid integrated into the chromosome. These findings are in contrast with the stable transconjugant 2D520(pUCD607) reported by Shaw and Kado (1986), where presence of the plasmid was confirmed by minscreen from bacteria reisolated from the plant. The possibility exists that in that particular strain, the plasmid was present in both free and integrated form, or was maintained in low copy number.

At this point in time it cannot be predicted over what period of time the "lux" genes will be stably expressed when 171LIIH-7 is growing in a cabbage field. Genes not under pressure often are turned off by organisms to reduce unnecessary energy load. 171LIIH-7 has shown no change in its ability to produce light after having been subjected to growth under different environmental conditions in the greenhouse or growth chamber. I believe that this transgenic organism is a fit pathogen for research purposes and poses no risk to the environment. A genetically engineered bioluminescent *X. c. campestris* already has been used in a field study by workers in Alabama (Shaw 1990).

When working with bioluminescent bacteria, temperature is an important factor to consider for meaningful results in disease studies. The expression of the "lux" genes from *V. fischeri* is temperature-dependent (Woodland and Hastings, 1985). The enzyme luciferase is most actively expressed in *V. fischeri* at 20 C and below and loses its function at higher temperatures, unless the NaCl concentration of the growth medium is raised (Waters and Lloyd, 1985). Strain 171LIIH-7 emits light at its brightest level between 16 - 25 C, but light production drops off progressively at temperatures above 25 C, although very low levels can still be detected by X-ray from agar cultures even at 34 C. This shows that luciferase is not completely inactivated at this temperature in this transformed *X. c. campestris* and is active as long as the bacteria are metabolically active. Bacteria can be grown at any permissible temperature for experimental use, and will simply have to be brought to room temperature from higher temperatures prior

to light detection and measurement (Shaw et al., 1987). Strain 171LIIH-7 biolumineses below 25 C too, but its growth rate is considerably reduced at lower temperatures.

This temperature dependence in light production is an important fact to keep in mind when a correlation is made between amount of light emitted and bacterial numbers, which is a direct relationship (Shaw and Kado, 1986). A separate standard for each different working temperature is therefore necessary. This applies also to the use of X-ray film to measure bacterial bioluminescence in plant tissues and relate it to bacterial numbers present. Shades of darkness on the exposed film reflect actual bacterial concentrations only when the temperature and exposure time are held constant. Exposure patterns on X-ray film from bioluminescent bacteria, which are growing at temperatures not permissive for full light production during the course of an experiment prior to X-ray exposure are dimmer, and do not accurately represent bacterial concentrations. When host-pathogen studies with glowing bacteria are done under uncontrolled conditions, it is advisable to incubate the experimental plants at a standard temperature, e.g. 25 C, for at least 2-4 hours prior to recording light. This assures full reactivation of the luciferase enzyme.

Temperature poses less of a problem in field studies, in which laboratory techniques for isolation and identification are applied. Night temperatures, even in Hawaii, usually permit visual detection of a bioluminescent pathogen in the field, or trace it with highly sensitive aerial devices (Shaw et al., 1987).

Moisture is an additional, highly important factor in accurate recording of light. Under conditions of saturating relative humidity, a film of moisture on the X-ray film inhibits proper exposure.

In conclusion, bacterial bioluminescence is a unique tool for studies of many aspects of the black rot of cabbage disease as long as the temperature effect on the enzyme and the problem with moisture are kept in mind. Bioluminescence in plant pathogens would be appropriate for the study of many other diseases.

CHAPTER III. FFECT OF HOST NUTRITION ON BACTERIAL INVASIVENESS

Introduction

Hawaii's farmers meet the demand for head cabbage in the local market, but serious losses are sometimes encountered from the systemic bacterial disease, black rot of cabbage. Continuous cropping and conducive weather conditions promote epidemics resulting in total crop losses. Control of black rot has been a problem (Alvarez and Cho, 1978), since *Xanthomonas campestris* pv. *campestris* (Xcc) is brought in on seed and rapidly spreads from infected plants by windblown water and by cultivational practices (Cook et al., 1952; Williams, 1980). Clean seed, careful sanitation methods, and sound cultural practices have been the means of controlling black rot.

With regard to cultural practices, numerous studies on the role of nutrition of the host plant response to invasion by parasites has been explored (Shear and Wingard, 1944; Palti, 1982; Huber and Watson, 1985). For diseases caused by xylem- invading *Xanthomonas* species the general conclusion has been reached that nutrient concentrations beyond the optimum for plant growth can decrease disease severity (Walker, 1951; Chase, 1989; Harkness, 1969; Thomas, 1965). Nitrogen has been studied most thoroughly, but the form of nitrogen provided to the host rather than the amount can have a greater effect on the disease process (Huber and Watson, 1974). The role of potassium and phosphorus in disease is not clear. Potassium at high concentrations seems to have an inhibitory effect on some *Xanthomonas* diseases (Huber and Amy, 1985; Matthee and Daines, 1967). However, in black rot of cabbage, potassium and phosphorus have not been shown to be important factors in the disease process (Walker and Gallegly, 1951).

The effect of increasing concentrations of nitrogen, potassium, and phosphorus on the invasiveness of the black rot pathogen is examined in this study. I have employed *X. c. campestris* tagged with "lux" to monitor the fate of the organism in the disease process. The bioluminescent pathogen is fully pathogenic and, in culture, can grow on different forms of nitrogen which are present in

the xylem of the cabbage plant. I will investigate the effects of nitrogen, potassium and phosphorus nutrition of the host as to the degree of invasiveness of *X. c. campestris.*

Materials and methods

Preparation of plant materials. Seeds of *Brassica oleracea* cv. *capitata*, G-C cross, were supplied by Dr. J. Cho (Dept. of Plant Pathology, University of Hawaii). Seeds were surface sterilized in 1% of 5.25% sodium hypochlorite for 10 minutes before sowing into steam-sterilized "Supersoil Potting Mix" (Rod McLellan Co.). As soon as the first primary leaf started to emerge, seedlings of approximately equal size were transplanted into plastic pots holding 27 inches^3 (443 cm^3) of a 1:1:1 mixture of vermiculite:perlite:peat. Vermiculite was omitted in experiments testing the effect of potassium only, because vermiculite releases potassium. The potting mix was amended with dolomite at 3 kg/m^3, and micronutrients were supplied in the form of Micromax (Sierra Chemical Co., Milpitas, CA) at the rate of 0.5 kg/m^3. Potassium and phosphorus were added as "Osmocote" pellets formulated for three-month release (Sierra Chemical Co., Milpitas, CA). Calculations for adequate amounts of potassium and phosphorus were based on a recommended rate of 5 kg/m^3 of a 19-6-12 "Osmocote" formulation (Waterer and Coltman, 1988). Nitrogen sources were ammonium nitrate, ammonium sulfate, or potassium nitrate, given to the seedlings in liquid form. A standard amount of 5.6 mg nitrogen for each seedling was derived from Hoagland's full-strength nutrient solution given every three days (Hoagland, 1933). When the amount of one element was varied, the amount of the others was held constant. Plants were watered by hand to eliminate any possibility of bacterial spread through splashing water drops. The plants were grown in a greenhouse under local environmental conditions. Maximum and minimum daily temperatures and relative humidity were recorded. The roof of the greenhouse was made from "Filon", a special greenhouse plastic, which is transparent to 95% daylight. On cloudless, hot days with temperatures above 30 C the cabbage plants were protected by 67% shade cloth.

Preparation of inoculum and inoculation method. The bioluminescent *X. c . campestris* 171LIIH-7 (this thesis) was grown on 523 agar (Kado, 1972) containing either spectinomycin at 40 ul ml^{-1} or kanamycin at 25 ug ml^{-1} at 29 C and routinely checked for bioluminescence before use. The wild type strain G-171 wild type was grown on 523 agar at 29 C. Bacteria from two-day old plate cultures were suspended in sterile water to $A_{600} = 0.1$ (approximately 10^8 cfu ml^{-1}). A notch (1 mm) was cut with a sharp razor blade into the tip of the midvein of the youngest fully expanded leaf and the first leaf below. The wound site was dipped into the inoculum for 20 seconds. The inoculated seedlings were placed into a plastic bag for 24 hours. The wounding method was preferred over spray-inoculation, because it was essential to have a single known site of ingress from which to monitor and measure the progress of the pathogen into the leaf. Early experiments included the wild type as control; later it was omitted due to space constraints and absence of observable difference in disease symptoms caused by both strains.

Monitoring the bacteria with bioluminescence. Location of the pathogen in the leaf was determined from leaf blots taken with Fuji-Medical X-ray film on days 3, 6, and 10 after inoculation. A paper leaf pocket containing a piece of preflashed X-ray film (Lasky, 1980) was placed around the inoculated leaf in the dark and attached with a large plastic clip. Care was taken to avoid moisture on the X-ray film by keeping all seedlings dry prior to exposure. Film was exposed for 12 hours at a temperature of 25-28 C.

Effect of nitrogen on bacterial invasion. Two types of experiments were designed to test nitrogen form and its effect on disease. In the first experiment, ammonium nitrate was applied to cabbage seedlings every three days in a range of six treatments (N in mg/pot for treatments 1 - 6: 0.56, 2.8, 5.6, 11.2, 16.8, 22.4). Each treatment consisted of six replications. After four weeks of treatment, one set of seedlings was inoculated with *X. c. campestris* G-171, the other with the bioluminescent transconjugant 171LIIH-7, and light measurements were taken at the described intervals. Visible lesion length was also recorded to compare symptoms produced by the transconjugant to those of

the wild type. Maximum and minimum temperature averages for the duration of the experiment were 30.9 C and 21.4 C respectively, and the average relative humidity was 63.5%. In the second experiment, ammonium sulfate and potassium nitrate were given as nitrogen sources. Amounts of nitrogen supplied to 8 replicates per treatment were in mg/pot for treatments 1 - 3: 0.56, 5.6, and 16.8 every three days for four weeks prior to inoculation. Average maximum and minimum temperatures were 29.5 C and 18.8 C respectively, and the average relative humidity was 64%. Light measurements were taken as described, visible lesions were measured, and the size of the infected leaves was recorded. At the conclusion of the experiments, the seedlings were ground for leaf analysis, and dry weights were recorded. Invasiveness of the pathogen under different fertilization regimes was calculated by measuring the lesion represented by the leaf blot. A qualitative evaluation was also done by comparing the relative darkness of the X-ray film exposures.

Effect of potassium and phosphorus on bacterial invasion. Working concentrations of each element were determined from a wide range of concentrations applied in a basic experiment, in which seedlings were fed 10 concentrations ranging from 0 to 7.98 g K per pot and 0 to 4.78 g P per pot. Final amounts used in subsequent experiments were chosen from the results, and were as follows for treatments 1 - 4: K in g/pot: 0, 0.057, 0.57, and 4.56, and P in g/pot: 0, 0.034, 0.34, and 2.04. Such high amounts were used because of the nature of the slow-release pellets and the relatively short treatment period. Nitrogen was applied as ammonium nitrate at the standard concentration of 5.6 mg N per pot every three days. The experimental procedure in three separate experiments followed the above described protocol

Effect of balanced fertilizer concentrations on bacterial invasion. This experiment complemented the studies on single element effects and examined the effect of "Osmocote" on cabbage seedlings. Varying the amounts of a balanced formulation will affect the plant growth status with respect to the relation of nutrition and disease. Working concentrations, based on a rate of 5 kg m^{-3} of a 19-6-12

formulation were 0.5x, 1x, 2x, 3x, 4x. The experimental procedure was the same as described above. Maximum and minimum temperature average in the greenhouse were 32 C and 22.5 C, respectively. The highest temperature was 34 C.

Bacterial growth in liquid culture as related to form of nitrogen in the medium. It was of interest to determine utilization of different nitrogen sources by the pathogen in culture. Growth curves were prepared for both the wild type and the transconjugant testing four nitrogen compounds: ammonium chloride, potassium nitrate, ammonium nitrate, and glutamine. Bacteria were grown to early stationary phase in 523 broth containing appropriate antibiotics, and inoculum was prepared by diluting the cultures to give a starting concentration of approximately 10^3 cfu ml^{-1}. Bacteria were grown in minimal medium (Miller, 1985) containing sucrose (10 ml/l of a 20% stock solution), methionine (4 ug ml^{-1}), and kanamycin (25 ug ml^{-1}) for the transconjugant. L-Methionine was added, because it enhances the growth of some bioluminescent bacteria (Doudoroff, 1942) and also many *Xanthomonas* species (Starr, 1945), although strain G-171 grows well in the absence of methionine on minimal medium with ammonium chloride and sucrose. Samples for dilution plating were removed at 0 hour and every four hours thereafter. Cultures were grown on a Lab-Line Orbit Environ shaker at 28-29 C. When potassium nitrate was the nitrogen source, samples for the nitrate test were removed at certain intervals, since *X. c. campestris* does not reduce nitrate (Dye, 1974).

Effect of sulfur and calcium on bacterial invasion. Seedlings were grown as described, but dolomite was omitted for the calcium test. Potassium and phosphorus were added as Osmocote pellets and nitrogen was given as liquid ammonium nitrate at 5.6 mg per pot every three days. A 1 M solution of calcium chloride was prepared and given in 25 ml per pot at the following concentrations: 0.1, 0.5, 1x, 2x, 3x, 4x, 5x, 6x, 7x, 8x. every three days. Sulfur was prepared as a 1 M solution of magnesium sulfate and sodium sulfate in a 3:1 ratio and fed in concentrations of 0.1, 0.5, 1x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, every three days. There were two plant replicates per treatment.

Results

Utilization of different nitrogen sources. The type of nitrogen form used did not result in obvious differences in the growth of bacterial cultures. Both the wild type and the transconjugant grew at a faster rate on ammonium chloride (Fig. 8), but at the same rate and to nearly the same density on potassium nitrate as on the other nitrogen sources, in spite of the classification of *Xanthomonas* as nitrate reductase negative (Dye, 1974). With methionine at a total concentration of 2.8 mg N (one tenth the amount of nitrogen supplied in minimal medium), nitrogen amount was insufficient to support growth of bacteria to 10^8 cfu ml^{-1}. A positive result of the nitrate test at 72 and 80 hours in the growth stage indicates that nitrate reductase was produced constitutively (Fig. 8). Low nitrite accumulation or rapid transition from NO_2 to N_2 prevents detections of NO_2, which is the basis of a positive test (McFadden, 1980).

Effect of ammonium nitrate on plant growth and bacterial invasiveness. Effects of the range of fertilizer concentrations are reflected in the growth of the seedlings (Fig. 9), dry weights, and leaf analysis (Table 4). Healthiest appearing plants had received 11.2 mg nitrogen every three days. At higher levels, leaves were darker, appeared thicker, and were slightly cupped. At the highest level of nitrogen, growth was somewhat reduced, which is confirmed by the average dry weight. As expected, the lowest amount of nitrogen reduced growth considerably. Seedlings were quite small, grew slowly, and their leaves were pale green. Symptoms from infection with the pathogen developed most rapidly on the nitrogen deficient seedlings in treatment 1, and quickly invaded the whole small leaf. Seedlings in treatments 2, 3, and 4 also developed typical black rot lesions, but lesion appearance on seedlings in treatments 5 and 6 was much slower with a reduction of chlorosis, the lesion representing a much smaller portion of the entire leaf. A comparison between lesion length measured visually and with X-ray film showed that the pathogen was far ahead of visual lesions, and the film exposures showed a fairly uniform pattern of infections in treatments 1 through 4 (Fig. 10, Table 4). In treatments 5 and 6, however,

Fig. 8. Growth of *X. c. campestris* strains G-171 and 171LIIH-7 with different nitrogen sources. Bacteria were grown at 28 C in minimal medium with sucrose and methionine. Nitrogen sources were ammonium chloride, ammonium nitrate, potassium nitrate, and glutamine. The arrow points to the point in the growth curve at which the nitrate test was positive.

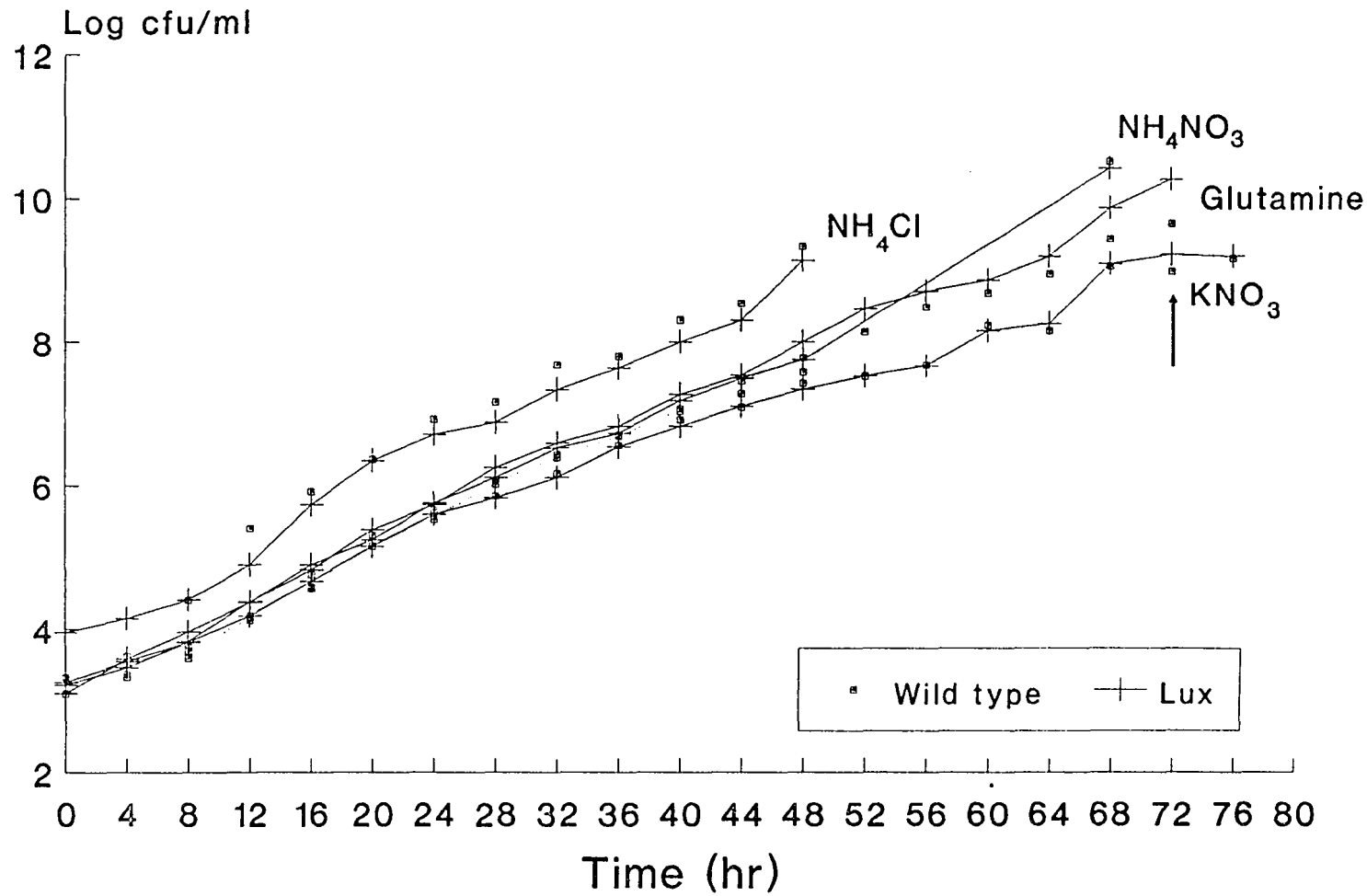

Log cfu/ml
12
10
8
6
4
2
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76 80
Time (hr)
NH_4NO_3
Glutamine
NH_4Cl
KNO_3
Wild type
Lux

Fig. 9. Effect of ammonium nitrate on the growth of cabbage seedlings. A) Seedlings representing growth with increasing nitrogen fertilization. Nitrogen applied in mg/pot every three days for treatments 1 - 6: 0.56, 2.8, 5.6, 11.2, 16.8, and 22.4. B) Comparison between treatments 1, 4, and 6.


A
T2

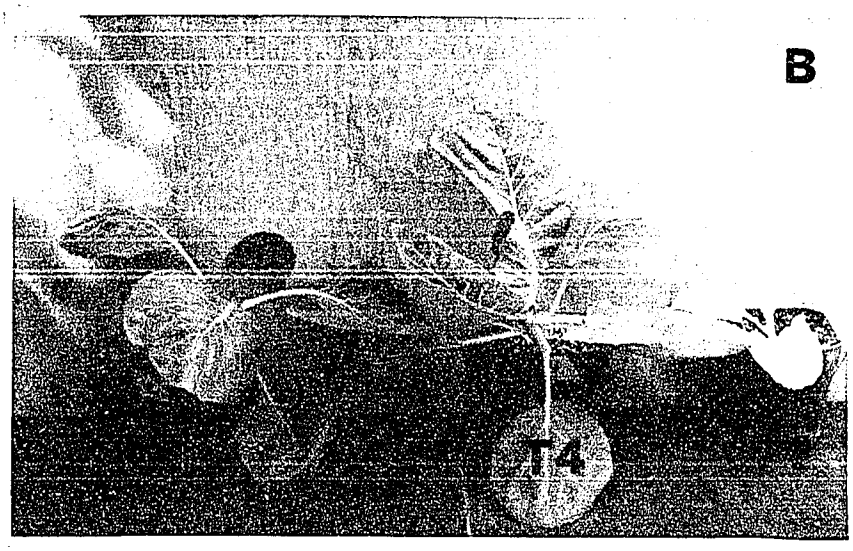

B
T4

Fig. 10. Leaf blots of strain 171LIIH-7 bearing "lux", infecting cabbage seedlings grown with different amounts of ammonium nitrate. X-ray film was exposed to infected leaves 10 days after inoculation. Numbers above photographs represent treatments 1 -6. Nitrogen was applied at 0.56, 2.8, 5.6 11.2, 16.8, and 22.4 mg/pot every three days.

NH_4NO_3

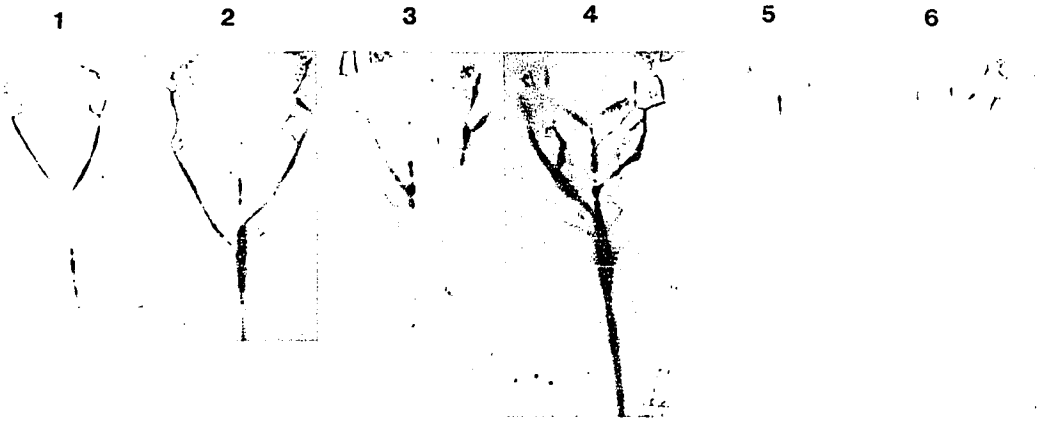


Table 4. The effect of ammonium nitrate fertilization on disease progression in cabbage seedlings

	Treatment					
	1	2	3	4	5	6
N in mg/pot [U]	0.56	2.80	5.60	11.2	16.8	22.4
Lesion length						
Visual (mm)	25.18[V]	40.67	41.17	37.42	18.41	23.17
Leaf blots with X-ray film (mm)	49.3a[W]	68.6b	83.5b	72.8b	50.7a	37.5a
Ratio of lesion length to leaf length (from leaf blots).	0.73a	0.71b	0.75b	0.74b	0.50a	0.39a
Diseased area (cm^2) (from leaf blots)	7.06a	14.20b	14.89b	16.43b	7.16a	7.35a
Leaf analysis						
%N/g dry weight	1.52[X]	2.60	2.83	4.02	5.80	5.87
Dry weights	0.46	1.27	1.81	3.47	2.73	2.47

U nitrogen amounts were supplied every three days.

V Values represent means calculated from 6 seedlings per treatment (2 leaves per seedling).

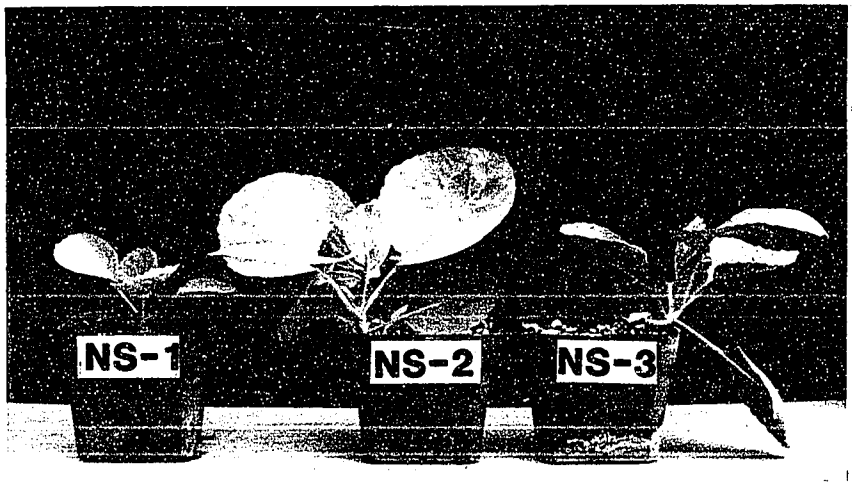
W Means within rows followed by the same letter are not significantly different (P = 0.05) as calculated by the Duncan-Waller K-ratio (LSD) Test and Duncan's Multiple Range Test - completely random design.

X Values represent means of 6 seedlings.

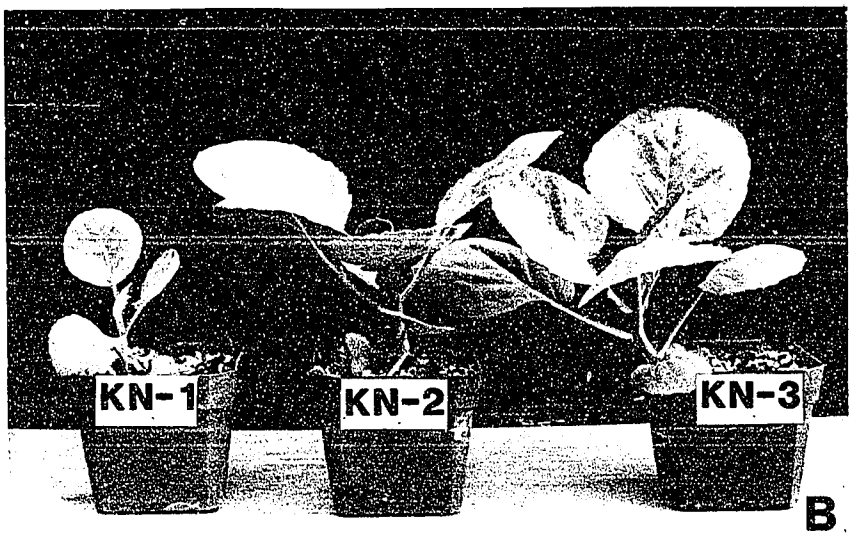
although most lesions were considerably reduced in both length and area, consistency in the replicates was lacking, e.g. four treatment-5 leaves and two treatment-6 leaves showed infections comparable to those in treatment-1 reduction of chlorosis leaves. Analysis of means of lesion length and total diseased area measured from leaf blots shows a significant difference for treatments 1, 5, and 6 from treatments 2, 3, and 4, according to the Duncan multiple range test. When the ratios of lesion length to leaf length were analyzed, treatments 5 and 6 differed significantly from the other treatments (Table 4). Leaf analysis showed that the nitrogen content in the leaves increased with increasing amounts given the seedlings, but reached a plateau in leaves of plants in treatment 5 (Table 4).

Effect of ammonium sulfate and potassium nitrate on bacterial invasion. Plant growth at the three different levels of nitrogen for both nitrogen sources is reflected in the photos (Figs. 11 and 12) and the dry weights (Tables 5 and 6). The lowest amount of nitrogen resulted in reduced growth regardless of nitrogen source. Potassium nitrate treatments 2 and 3 produced larger and healthier appearing seedlings than ammonium sulfate treatments 2 and 3 (Figs. 11 and 12), and leaf analysis showed amounts of N to be adequate for cabbage seedlings (3-5%)(Nieuwhof, 1969; Mills and Wolf, 1991). Ammonium sulfate fertilization resulted in 1.27 percentage units more nitrogen in seedlings than did potassium nitrate fertilization. Average visual lesion length differed slightly under the two nitrogen treatments. In seedlings receiving potassium nitrate, bacteria progressed somewhat further than in ammonium sulfate fed seedlings in treatments 1 and 2, but at the high nitrogen level there was no apparent difference (Table 5). Overall invasiveness of the bacteria measured by qualitative X-ray evaluation was more pronounced in potassium nitrate fed seedlings and was reduced at the highest concentration (Fig. 13). The X-ray photos suggest a reduction in lateral spread of the bacteria in treatment 3 of potassium nitrate, but in half of the infected leaves the pathogen had spread down into the petioles. Means of lesion length, ratios of lesion length to leaf length, and percent leaf area diseased were analyzed by the Duncan-Waller multiple range test, and significant differences for the different nitrogen fertilization regimes were observed (Table 6). There was a significant increase in diseased area at the low nitrogen treatment (T1)

Fig. 11. Growth of cabbage seedlings with different amounts of nitrogen. A) Seedlings fertilized with ammonium sulfate every three days with 0.56, 5.6, and 16.8 mg/pot for treatments 1 -3, respectively. B) Seedlings fertilized with potassium nitrate every three days with 0.56, 5.6, and 16.8 mg/pot for treatments 1 - 3, respectively. Fertilization period was four weeks.



A



B

Fig. 12. Comparison of seedling growth from fertilization with ammonium sulfate to potassium nitrate. A) Treatment 1, 0.56 mg nitrogen/pot; B) treatment 2, 5.6 mg nitrogen/pot; C) treatment 3, 16.8 mg nitrogen/pot. Treatments were given every three days for four weeks.

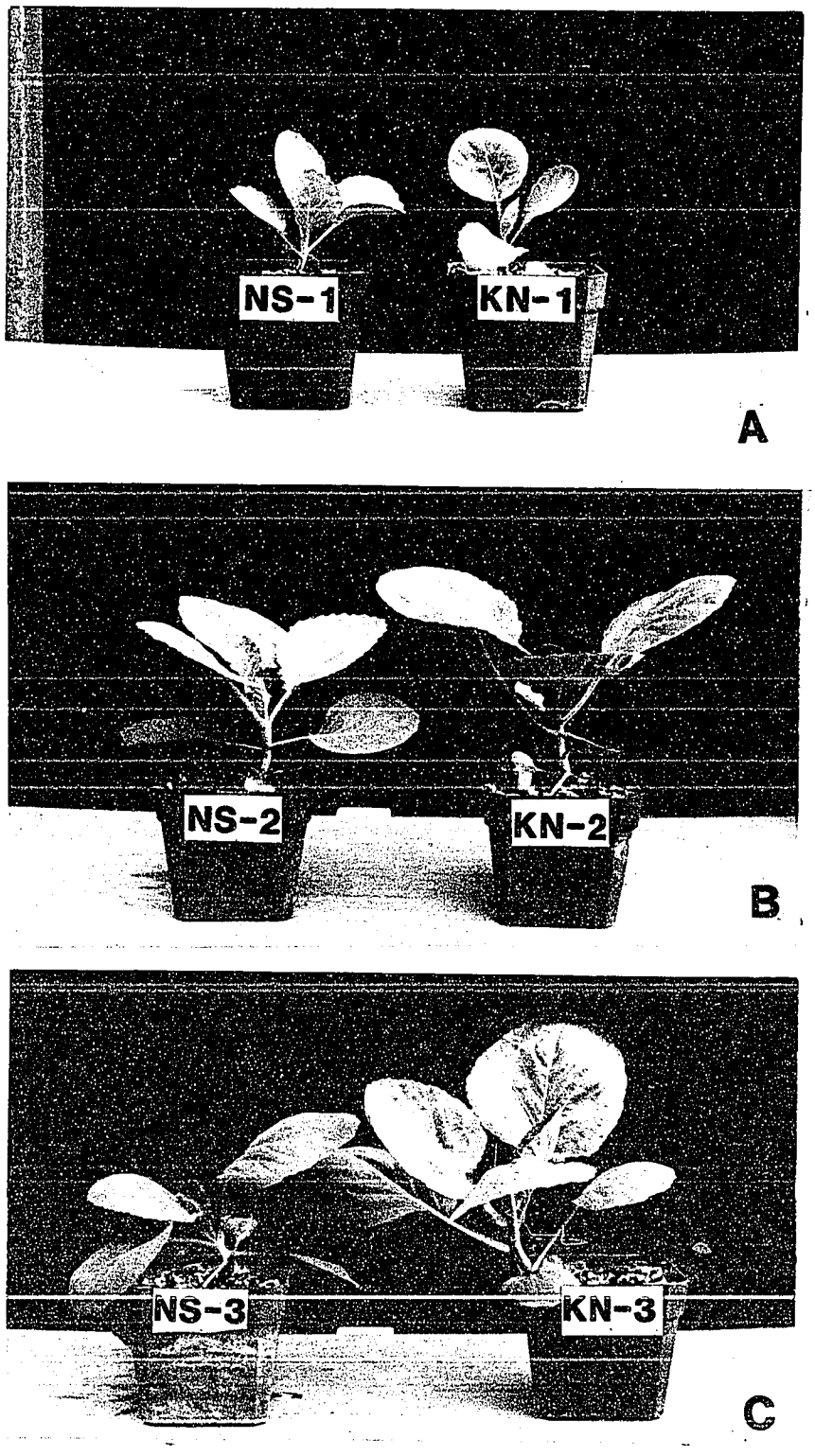
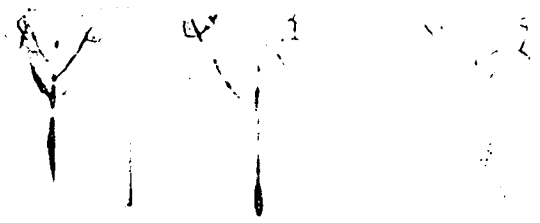

NS-1
KN-1
A
NS-2
KN-2
B
NS-3
KN-3
C

Fig 13. Leaf blot patterns of 171LIIH-7 infections in cabbage seedlings grown with different nitrogen fertilization. Ammonium sulfate and potassium nitrate were applied in mg/pot for treatments 1 -3: 0,.56, 5.6, and 16.8. Ammonium nitrate treatment 2 consisted of 5.6 mg/pot nitrogen. Treatments were given every three days for four weeks, and exposures were taken 10 days after inoculation.

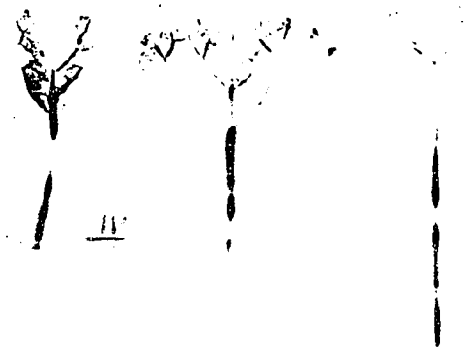
$(NH_4)_2SO_4$

1 2 3



KNO_3

1 2 3



NH_4NO_3

2

Table 5. Comparision of different nitrogen fertilization regimes to disease progression

	Nitrogen source			
	Ammonium sulfate	Potassium nitrate	Ammonium nitrate [a]	Ammonium nitrate [b]
Treatment 1 (0.56 mg N)				
visible lesion length (mm)	19.80[c]	22.40	25.18[d]	-
leaf blots lesion length (mm)	51.3	55.5	49.3	-
Ratio lesion length to leaf length (leaf blots)	0.72	0.75	0.73	
% area diseased (leaf blots)	24.95	28.07	-	-
% leaf N (dry basis)	1.42	1.44	1.52	-
dry weight (g)	0.467	0.418	0.457	-
Treatment 2 (5.6 mg N)				
visible lesion length (mm)	28.90	35.90	41.17	26.50
leaf blots lesion length (mm)	65.1	95.2	83.5	62.5
Ratio lesion length to leaf length (leaf blots)	0.61	0.81	0.75	-
% area diseased (leaf blots)	12.77	14.23	-	10.55
% leaf N (dry basis)	2.60	2.45	2.83	2.67
dry weight (g)	2.142	2.360	1.811	1.964
Treatment 3 (16.80 mg N)				
visible lesion length (mm)	23.40	23.20	18.42	-
leaf blots lesion length (mm)	49.9	77.6	50.7	-
Ratio lesion length to leaf length (leaf blots)	0.42	0.71	0.50	-
% area diseased (leaf blots)	8.40	7.75	-	-
% leaf N (dry basis)	6.12	4.85	5.80	-
dry weight (g)	2.060	3.240	2.731	-

- = not done
a = experiment done at a different time (TABLE 1)
b = treatment 2 done only as a reference for the previous experiment.
c = values represent calculated means from 8 seedlings (2 leaves per seedling)
d = values represent calculated means from 6 seedlings (2 leaves per seedling)

Table 6. Analysis of means obtained from nitrogen fertilization

Nitrogen source	Treatment N in mg/pot	%N/g dry weight	dry weight	Measurements from leaf blots [x]		
				lesion length (mm)	Ratio lesion length to leaf length	% area diseased
$(NH4)_2SO_4$	1 - 0.56	1.42a[y]	0.468a	51.3a	0.78bc	24.95b
	2 - 5.60	2.60b	2.142bc	65.1bc	0.61b	12.77a
	3 - 16.80	6.12d	2.063b	49.9a	0.42a	8.40a
KNO_3	1 - 0.56	1.44a	0.418a	51.3a	0.75bc	28.07b
	2 - 5.60	2.45b	2.365c	95.2c	0.81c	14.23a
	3 - 16.80	4.85c	3.240d	77.3bc	0.71bc	7.75a

x = mean values represent 8seedlings (2 leaves per seedling)

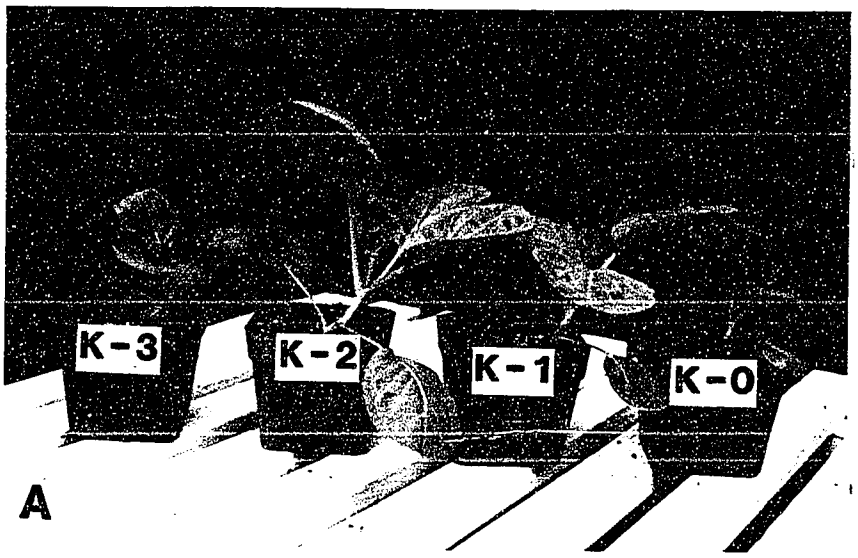
y = mean values followed by the same letter in each column do not differ significantly (P =0.05) as determined by Duncan's multiple range test, and Duncan and Waller K-ratio test.

for both ammonium sulfate and potassium nitrate, and a significant decrease with increasing amounts of nitrogen. A statistically significant difference between the two nitrogen treatments was also shown for lesion lenghts and ratios of leasion length to leaf length.

Effect of potassium and phosphorus on bacterial progression. When either potassium or phosphorus was omitted from formulations (T1) in the first two experiments, seedlings were small and grew very poorly (Fig.14 and 15). The seedlings grown with low amounts of potassium (T2) were also small and had cupped leaf edges, whereas seedlings grown with phosphorus at T2 amounts were smaller compared to those growing on higher amounts, but showed no apparent deficiency. The recommended amount of both K and P produced the healthiest plants (T3). At the highest application (T4) plant growth was inhibited to a certain extent, but the effect was more obvious for seedlings treated with potassium than for those receiving phosphorus (Fig. 14). Whereas most of the phosphorus T4 plants were healthy, about half of the K4 plants had cupped leaves. Results from experiments I and II were similar. Under the maximum prevailing temperatures (32.4 C) in experiment 3, plant response to treatments 2 and 3 for either K or P did not differ from seedlings grown at 28 C. However, when the highest amount of the elements (T4) was supplied, two effects from the higher temperature were seen: 1) the plant growth inhibition by high amounts of K (T4) seen at 28 C was negligible at 32.4 C (Fig. 14 and 15); 2) with high phosphorus (T4) growth was enhanced at 32.4 C as opposed to reduced at 28 C (compare Figs. 14 and 15).

Lesions developing on seedlings deprived of either K or P (T1) were generally not as severe, which was also indicated by visual lesion length and by X-ray photographs (Table 7; Fig. 16). At high concentrations (T4), lesions were also slightly reduced. Disease was most pronounced at optimum plant vigor (T3). Seedlings grown with minimal amounts of K (T1) showed no disease reduction, but a low supply of P (T1) resulted in slightly reduced lesions. (This was confirmed by the X-ray patterns (Fig. 16).

Fig. 14. Growth of cabbage seedlings with different amounts of potassium and phosphorus after four weeks at 28 C. A) K treatments 1 - 4. Amounts of Osmocote pellets supplied in g/pot: 0, 0.057, 0.57, and 4.56.
B) P treatments 1 - 4. Amounts of Osmocote pellets supplied in g/pot: 0, 0.034, 0.34, and 2.04.


K-3
K-2
K-1
K-0
A

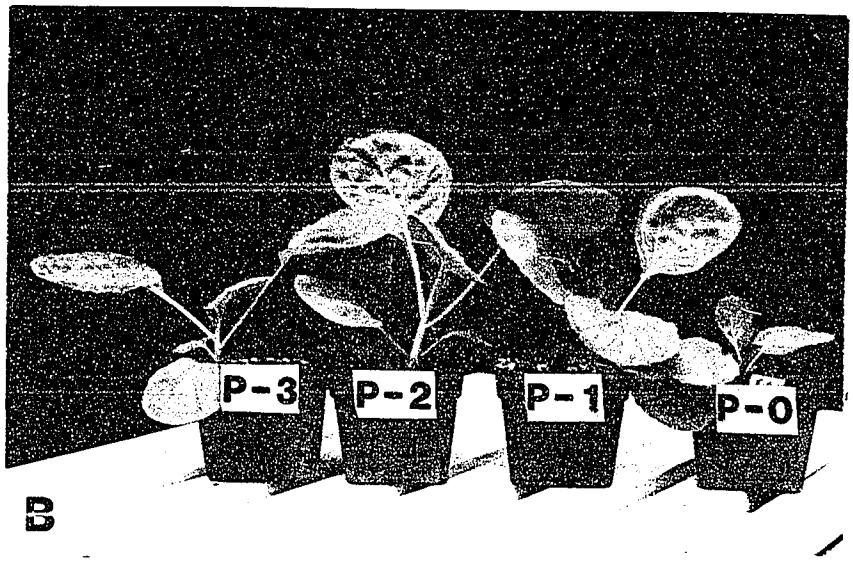
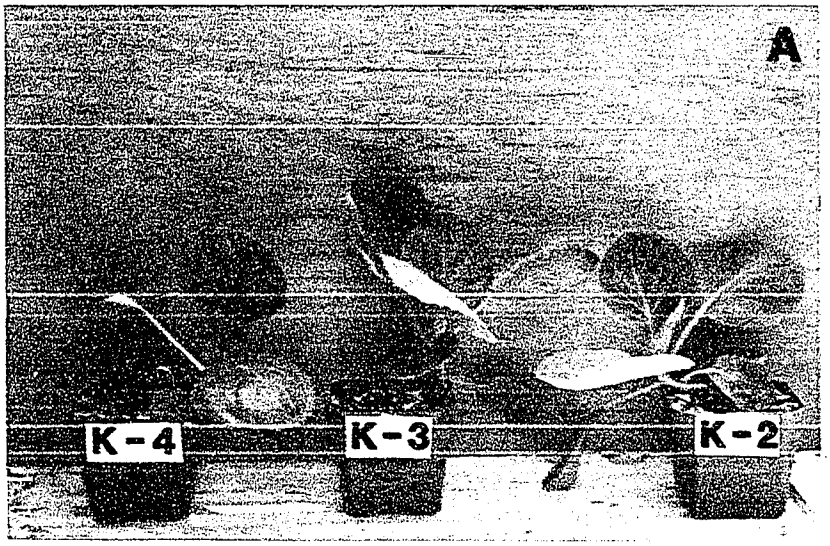

P-3
P-2
P-1
P-0
B

Fig. 15. Growth of cabbage seedlings with different amounts of potassium and phosphorus after four weeks at 32.4 C. A) K treatments 2 - 4. Amounts of Osmocote pellets supplied in g/pot: 0.057, 0.57, and 4.56. B) P treatments 2 - 4. Amounts of Osmocote pellets supplied in g/pot: 0.034, 0.34, and 2.04.


A
K-4
K-3
K-2

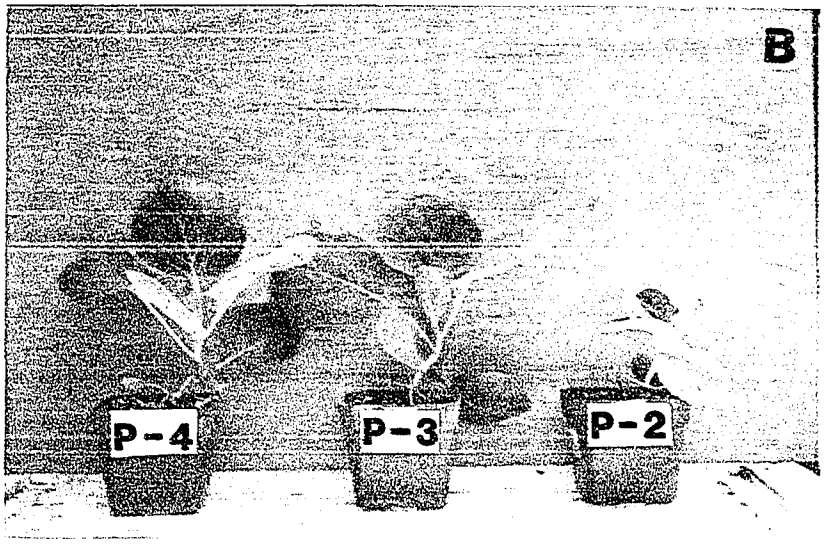

B
P-4
P-3
P-2

Fig. 16. Leaf blot patterns of 171LIIH-7 infections in cabbage seedlings grown with different amounts of potassium and phosphorus at 28 C. Amounts of Osmocote pellets supplied in K treatments 1 - 4 in g/pot: 0, 0.057, 0.57, and 4.56, and in P treatments 1 - 4: 0, 0.034, 0.34, and 2.04.

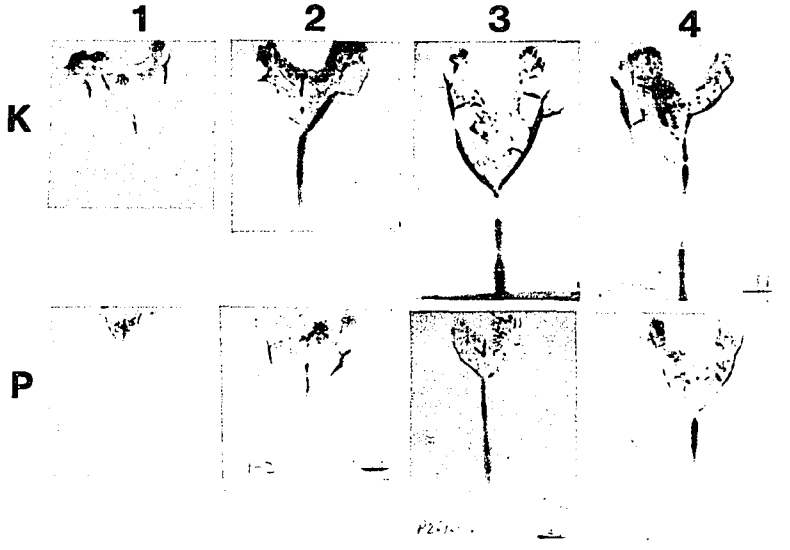

1
2
3
4
K
P

TABLE 7. Effect of potassium and phosphorous fertilization on lesion length

	Lesion length in mm							
	Expt. I (January)[a]			Expt. II (March)[b]			Expt. III (July)[c]	
	LIIH-7		Wild type	LIIH-7		Wild type	LIIH-7	
Treatment	Visual	X-ray	Visual	Visual	X-ray	Visual	Visual	X-ray
Potassium								
T-1	10.60	ND	12.50	15.80	41.42	12.75	ND	ND
T-2	13.65	24.00	17.35	31.00	64.50	26.33	24.50	54.29
T-3	20.25	39.50	21.25	31.80	66.92	22.10	28.75	51.86
T-4	17.65	24.38	18.85	19.90	57.25	21.30	37.00	66.25
Phosphorous								
T-1	10.50	26.17	3.00	10.40	25.92	4.90	ND	ND
T-2	9.10	25.62	16.45	21.30	50.08	23.60	24.50	36.57
T-3	25.00	48.25	25.30	26.70	58.92	23.90	28.00	39.37
T-4	17.60	19.87	18.60	18.85	46.50	20.10	30.87	62.13
Temperature averages °C								
maximum		26.40			28.22			32.36
minimum		20.24			18.58			23.00
highest		31.00			31.70			35.00
lowest		16.00			14.50			20.00

a = measurements represent averages of 8 leaves from 4 replicate plants.
b = measurements represent averages of 12 leaves from 6 replicate plants.
c = measurements represent averages of 8 leaves from 8 replicate plants.
d = amounts of potassium given in g/pot : T-1 through T-4 = 0, 0.57, 0.57 and 4.56 .
e = amounts of phosphorous given in g/pot : T-1 through T-4 = 0, 0.034, 0.34 and 2.04.
ND = No data.

Table 8. Effects of potassium and phosphorus fertilization on tissue analysis of *Brassica oleracea*

Treatment	Osmocote pellets per 8-cm pot (g)	Percentage dry weight						Parts per million					
		N	P	K	Ca	Mg	Na	Mn	Fe	Cu	Zn	B	Al
Expt. II*													
P-1	0.000	4.67	0.11	4.66	2.36	0.84	0.17	403	179	71	222	53	94
P-2	0.034	3.54	0.17	5.12	1.97	0.78	0.31	334	85	19	102	56	36
P-3	0.340	3.39	0.52	4.86	2.17	0.90	0.52	414	73	21	126	54	88
P-4	2.400	3.51	0.64	4.71	2.06	1.05	0.37	518	96	23	244	56	117
K-1	0.000	5.17	0.69	1.01	2.17	1.46	2.94	351	12	21	136	66	126
K-2	0.057	4.52	0.69	2.42	1.91	1.27	1.95	365	58	23	140	73	130
K-3	0.570	3.37	0.51	3.58	1.82	0.98	0.60	339	33	17	106	47	118
K-4	4.560	3.18	0.53	18.22	0.83	0.47	0.22	223	79	22	189	49	141
Expt. III													
P-2	0.034	3.44	0.15	5.66	2.12	0.83	0.51	297	45	15	133	55	141
P-3	0.340	3.18	0.46	6.14	2.30	0.99	0.62	282	59	15	105	50	173
P-4	2.400	3.03	0.62	6.31	2.34	0.97	0.48	422	49	19	330	47	160
K-2	0.057	3.46	0.45	1.81	1.99	1.18	2.38	307	37	16	129	52	159
K-3	0.570	3.21	0.45	5.18	2.03	1.17	0.74	326	44	15	131	47	168
K-4	4.560	3.41	0.51	10.34	0.96	0.54	0.25	235	40	16	192	50	141

* Expt. II was done at 28C; Expt. III was done at 32.4C

Visual lesion length measurements at 28 C and 32.4 C did not suggest a difference in infection severity, nor did X-ray exposure pattern, which had limited use for the 32.4 C experiment due to very much reduced light production by the bacteria.

Effect of magnesium sulfate and calcium chloride on bacterial invasiveness. There was no visible effect of magnesium sulfate on either plant growth or lesion development from any of the treatments. Calcium chloride reduced plant vigor at the highest concentrations (T6-T10). Black rot infection was minimal at the high concentrations for T7-T10 as judged by eye. Seedlings which had received no calcium chloride had the most pronounced lesions. The X-ray film leaf blots were not very clear, but also showed lesion reduction at the higher levels of calcium chloride. The effect of these two salts was not pursued further.

Effect of Osmocote on bacterial invasiveness. Seedling response to Osmocote fertilization was very poor. Only at the lowest concentration, which was 50% of the recommended rate, were the plants healthy. Growth was severely restricted in all other treatments, leaves were badly cupped, and seedlings treated with amounts above the recommended rate wilted readily even under adequate water supply. Seedlings which had received the highest amount of Osmocote (4x) could not be inoculated due to poor condition. Leaf blot patterns from infections in treatments 0.5x, 1x, and 2x Osmocote were very dim, and with 3x the recommended rate of the fertilizer, only one leaf out of 16 had a very small lesion. The average maximum and minimum temperatures during the experiment were 32 C and 22.5 C, respectively.

Discussion

In some host-pathogen systems, vigorously growing plants under high nitrogen fertilization regimes are more susceptible to invasion by the pathogen (Spencer and McNew, 1938; Chase, 1989). My experiments with varying amounts of ammonium nitrate do not support such a premise for black rot of cabbage. At low levels of nitrogen, which resulted in stunted growth of the seedlings and obvious nitrogen starvation (Fig. 8, Table 4, Ch. III), disease was very severe. Within 10 days, lesions extended over the entire leaf, and the pathogen had progressed into the stem. The infection patterns recorded on X-ray film for treatments 1 - 4 were similar, but depicted markedly reduced infections from higher amounts of ammonium nitrate in treatments 5 and 6, where most of the chlorosis usually associated with typical black rot lesions was absent (Fig.10,Ch.III).

These observations are in agreement with results from similar experiments by Walker and Gallegly (1951), in which the relationship of nutrition to the severity of black rot was examined; disease was reduced as nitrogen was supplied in increasing amounts. In this earlier study, nutrients were supplied to the seedlings in liquid formulation at a constant rate, but in my experiment only nitrogen was applied in liquid form, whereas potassium and phosphorus were mixed into the potting medium as slow-release pellets, and micronutrients were added in powder form. Since the rate of release of elements from slow-release pellets is temperature-dependent, K and P probably became available to the seedlings at slightly varying rates (Tamimi et al., 1989) over the course of the experiment. However, the difference in experimental procedure did not change the results, which support the observations that increasing nitrogen decreases black rot of cabbage (Walker and Gallegly, 1951).

The reduction of disease at higher levels of nitrogen fertilization was not dependent on nitrogen form as suggested by Huber (1974) for a number of diseases; rather I found that disease reduction was similar for all three nitrogen compounds tested. In addition, it can be stated without reservation, that insufficient nitrogen, either in the ammonium or nitrate form, is related to increased disease severity. The percentage of diseased leaf area in seedlings grown under low nitrogen ammonium sulfate and

potassium nitrate fertilization was significantly greater, and decreased as nitrogen amounts increased (Tables 5 and 6, Ch. III). In the ammonium nitrate experiment, total lesion size resulting from the lowest amount of nitrogen did not differ significantly from those in treatments 5 and 6; however, it has to be pointed out that these values do not take into account the differences in leaf size. The leaves on seedlings grown under the high nitrogen regimes were four times as large as those on seedlings growing under low nitrogen. This is reflected by the values for average dry weights of the seedling (Table 4, Ch. III). Therefore, the pathogen became systemic much more rapidly in the low nitrogen seedlings.

Results from *in vitro* experiments, in which both the transconjugant and the wild type were tested for utilization of different nitrogen compounds in liquid culture, are relevant here. The growth curves confirmed that the bacteria could use nitrogen from the different N compounds almost equally efficiently in the minimal medium (Fig. 8, Ch. III), although this was not expected from the described characteristics of the genus *Xanthomonas* (Dye, 1974). However, the strains grew at an accelerated rate on ammonium chloride in comparison to potassium nitrate. Can this be related to bacterial growth in the host plant which has been fertilized with increasing amounts of either of these two nitrogen forms? In the xylem, nitrogen is available in several forms, as the ammonium ion from passive uptake from the roots, as nitrate, and as products of $NH4^+$ metabolism from root synthesis of amino acids (Lewis, 1968; Oaks and Hirel, 1985; Costigan and Mead, 1987; Gardner and Ross, 1989; Abrol, 1990). The ratio of these ions in the xylem fluid is dependent on many factors, which include availability and uptake of the ion, competition with other ions present, plant metabolism, and environmental factors. Without data on solute composition of the xylem fluids, it is difficult to evaluate an effect of any one of the nutrients on pathogen growth. We can only draw conclusions from visual symptoms, and leaf blot and leaf analysis.

In this study, the use of bioluminescence revealed some interesting facts that showed that visual evaluation in black rot disease expression does not provide us with a true picture of ongoing host-pathogen interactions. This discovery was particularly pertinent for the ammonium sulfate and potassium nitrate fertilization studies. Visual lesion lengths on seedlings growing with the recommended

rates of the two nitrogen forms (T2) pointed to a faster rate of progression of the pathogen into the leaf, but to a slower rate at both the very low and the high levels of nitrogen (Table 5, Ch. III). However, when the values for percentage of diseased area are calculated from the leaf blot patterns and analyzed in the Duncan-Waller test, it becomes apparent that disease was significantly greater in the nitrogen starved plants (T1) (Table 6, Ch. III). These values also established that bacterial invasiveness was inhibited with increasing nitrogen levels for both $(NH_4)_2SO_4$ and KNO_3. Without the leaf blot data I could have arrived at the erroneous conclusion that low nitrogen in the form of ammonium or nitrate is related to less severe disease than at recommended rates of these nitrogen forms. For the same experiment, another interesting observation can be made from the leaf blot patterns. The reduction of the percentage of diseased area in plants growing with high amounts of ammonium sulfate corresponds to a reduced downward spread of the bacteria (Fig. 13, Ch. III). In contrast, the reduction in diseased area in seedlings fed with high amounts of potassium nitrate corresponds to a concentration of the bacteria in the vascular bundle of the midvein and an increased downward movement by the bacteria along the entire length of the petiole.

Two different processes appear to be affected as a direct result of different forms of nitrogen fertilization: 1) the spread of the bacteria throughout the leaf area, and 2) the invasion of the pathogen directly into the petiole without significant spread into other leaf veins. I speculate that the basis for this observation is related to the growth rates of the pathogen in xylem fluids affected by different nitrogen fertilizations for the seedlings. If the xylem fluid of KNO_3 fertilized plants provides a preferred environment for the growth of the pathogen, generation time would be reduced, and bacterial masses would increase rapidly. The larger xylem vessels of the midvein could then accommodate these masses more readily. On the other hand, if under excessive ammonium sulfate fertilization ammonium accumulates in the xylem fluid, because the plant cannot provide the large amounts of carbohydrate necessary for rapid NH_4^+ assimilation, ammonium toxicity occurs. Inhibitors may be produced under these conditions which slow bacterial progression, and the pathogen would also invade the smaller veins,

where inhibitors are diluted. I am saying that we are dealing with rapid mass movement into the midvein with high KNO_3 fertilization, and slower progression into the midvein with high $(NH_4)_2SO_4$ fertilization with concomittant spread into the small leaf veins. Qualitative and statistical evaluation of the leaf blots indicates that a rapid downward movement in the midvein occurred in the KNO_3 treated plants vs the $(NH_4)_2SO_4$ treated seedlings in treatment 3 (Fig. 13, Ch. III). Reduced area of leaf lesions in plants fertilized withNO_3-nitrogen does not necessarily indicate that invasiveness of the pathogen is restricted, because the bacteria have invaded the petiole and progressed into the stem.

It is difficult to relate seedling vigor to bacterial invasiveness from results obtained in the different nitrogen experiments as well as from potassium and phosphorus fertilization. Seedlings fertilized with the high amounts of potassium nitrate were larger and sturdier than seedlings which received the same amount of nitrogen in the ammonium form. These smaller plants may have suffered from ammonium toxicity, which has been described as one of the disadvantages of ammonium fertilization (Lewis, 1986). Cabbage, along with many crop plants, gives higher yields with ammonium nitrate fertilization compared to other nitrogen formulations (Smith and Hadley, 1988). When nitrogen was supplied in the field in the form of sodium nitrate, ammonium nitrate, and ammonium sulfate, the yields in tons per acre were identical for the two forms of nitrate fertilization, but 2.3% lower for the ammonium sulfate fertilization (Nieuwhof, 1969). I strongly suspect that in my experiments with ammonium and nitrate fertilization, the inhibition of seedling growth from high ammonium sulfate (T3) could be due to ammonium toxicity on the one hand and a reduction of potassium uptake on the other (Lewis, 1986). Nitrate in contrast to ammonium can accumulate as a storage compound in leaves and other plant parts, and therefore does not become toxic at high uptake rates. Furthermore, the overall beneficial effect on plant growth at levels of potassium nitrate given in treatments 2 and 3 might be due to the additional amounts of potassium which became available from the nitrate fertilization. Potassium is especially important for cabbage seedlings during the first few weeks of growth, at which time potassium levels in the seedling rise rapidly, and plant weight rises steadily as potassium levels supplied

rise until a plateau is reached (Costigan and Mead, 1987). The largest potassium nitrate fertilized plants had considerably reduced lesions and also had the lowest percentage of diseased leaf area as measured by X-ray film (Tables 5 and 6, Ch. III). In a crop situation, such data could lead to the erroneous conclusion that disease is least severe in the most vigorously growing plants, when in fact the bacteria were rapidly progressing into the leaf petiole of these plants without producing large lesions, as was discussed above.

Potassium and phosphorus fertilization had different effects on plant growth and disease. The results in the two separate experiments were influenced by ambient temperatures, and the enhancement of seedling growth at the highest levels (T4) at 32.4 C for both K and P can be attributed to an accelerated release of the nutrients from Osmocote and uptake into the plant under the higher temperature. Plant analysis confirmed that the percentage of P in T4 plants grown at 32.4 C was sufficient, but not high, whereas K content was excessive (Jones et al., 1991). This explains the obvious vigor of the phosphorus fertilized seedlings over the potassium fertilized seedlings in treatment 4 (Figs. 14 and 15, Ch. III). At very high K concentrations in the root zone cation competition with calcium, magnesium, and sodium would reduce uptake of the latter elements by the plants (Hara et al.), and this was confirmed by the plant analysis (Table 8, Ch. III). Phosphorus, on the other hand, does not compete with other cations and did not inhibit plant growth at the higher temperature. Growth enhancement at higher temperature was not attributed to any single nutrient.

Potassium and phosphorus were not related to disease development in these experiments, which is in agreement with Walker and Gallegly's work (1951) on the effect of phosphorus on black rot of cabbage, but is different from potassium studies on other *Xanthomonas* species, which are reported in summaries by Huber and Arny (1985) and by Palti (1981) to be decreased by potassium fertilization. Chase (1989) and McGuire (1991) also described disease inhibition by *Xanthomonas* leaf pathogens at high levels of K, but this observation comes from experiments in which plants were fed with a combination of high nitrogen and high potassium. In my experiments, the nitrogen concentrations in the plants growing under high levels of either potassium or phosphorus were sufficient, but not high.

In general, no significant difference in lesion measurements was seen from K or P fertilization as analyzed by the Duncan-Waller multiple range test (not shown). However, at 32.4 C vs 28 C at the highest P treatment, which produced the largest, sturdiest seedlings, significantly longer lesions were recorded with leaf blot analysis (Table 7, Ch. III). This observation confirms similar results by other researchers, where an increase in disease occurred with increasing amounts of phosphorus in *E. stewarti* and *X. pelargonii* infections (Spencer and McNew, 1937; Kivilaan and Scheffer, 1958).

The value of X-ray photography to assess the extent of the infection by the pathogen was reaffirmed in the K and P experiments, because it also reflected a qualitative difference in disease from these two nutrients. Patterns interpreted from black rot infections at 28 C indicate a higher number of bacteria in the potassium versus the phosphorus treated seedlings (Fig. 16), since amount of light emitted is directly proportional to numbers of bacteria (Shaw and Kado, 1986), and therefore the darkness of the exposure on the film is related to bacterial concentrations. A stronger exposure (darker shading) is correlated to an increase in bacterial populations.

Bioluminescence in combination with leaf analysis also provided a clue in the disease development under K and P fertilization. When K or P was omitted from the fertilization (T1, Table 7, Ch. III), the leaf blot patterns confirmed the visual observations that lesion size was much reduced on these seedlings, especially in the case of phosphorus. These results are in direct opposition to the results from low nitrogen fertilization, where disease was increased compared to high nitrogen fertilization. The leaf analysis values for K and P could explain the inhibitory effect on the bacteria growing in seedlings which received no K or no P.

In the leaf analysis (Table 8, Ch. III) low values for K and P in the T1 seedlings were associated with somewhat high nitrogen content, 5.17% and 4.67%, respectively. A comparison of these nitrogen percentages with those on Table 4 show that they fall into the transition zone where a reduction in disease occurred at higher levels of N from ammonium nitrate fertilization (between treatments 4 and 5). I

therefore postulate that the disease reduction I have observed in all of these experiments may be attributed to an effect of increased amounts of leaf nitrogen.

Leaf analysis in these experiments revealed that P content was merely sufficient, but not high (Jones et al., 1991), and that amounts calculated for "high" P treatments (T4) were insufficient. Therefore I cannot compare my results to results obtained by Walker and Gallegly (1951) who used excessively high P levels.

The described findings have certain implications for field recommendations only in a limited sense. The rate of nutrient uptake, which is dependent on complex interactions between ions and microbiota in the soil rhizosphere, is not reflected in the artificial growth medium used in these experiments. It is not the purpose of this study to make field recommendations, but I think that the results indicate that starving the plant by withdrawing nitrogen will benefit only the pathogen, not the plant. It is my opinion that as far as plant vigor in relation to disease severity is concerned, ammonium nitrate is the most effective and preferred nitrogen fertilization for cabbage. In vigorously growing plants with slightly increased amounts of ammonium nitrate, where a reduction in lesion size was noticed, the early removal of an infected leaf would prevent the pathogen from becoming systemic.

Considering the improved growth of cabbage seedlings under potassium nitrate fertilization and the reduced lesions in black rot infections at high amounts, this nutrient might seem preferable to ammonium fertilization, but, as indicated by the leaf blot patterns, a problem may arise if KNO_3 amounts are increased above currently recommended amounts. The pathogen may multiply mainly in the vascular bundle of the midvein and petiole (Fig. 13, Ch. III), producing latent infections which would be undetected. On the other hand, spread in the field to surrounding plants might be reduced due to small lesion size, because less inoculum is available at leaf margins. It would have to be established whether invasion of the pathogen into other leaves will eventually take place, or if the bacteria remain restricted to the vascular bundles of the stem.

The importance of the rate at which nutrients are supplied to the seedlings was shown in the experiment with Osmocote. The poor condition of seedlings treated with the recommended levels and above, was demonstrated by extremely poor plant growth. The unexpected detrimental effect of the recommended amounts of fertilizer on the seedlings was due to rapid nutrient release at the high temperatures during the experimental period, which averaged 32 C for at least 6 hours of the day. Since release of elements from Osmocote is temperature dependent, approximately 65% of nitrogen would have been released by the time the seedlings were inoculed (Tamimi et al., 1989). In the process of root uptake of both nitrogen and potassium pH changes occur (Mills, 1991) which in turn influence uptake of others ions. An imbalance of elements causes changes in the plant metabolism, e. g., potassium promotes synthesis of organic nitrogen substances, and when the plant is deficient in this element, nitrogen accumulates in the sap and may influence the growth of a pathogen in the xylem (Shear and Wingaard, 1944). Phosphorus was found to increase ammonium and decrease nitrate nitrogen (Younts and Musgrove, 1958). Nitrogen starvation increases carbohydrate production, which may affect some pathogens (Aslam et al., in Oaks and Hirel, 1985). The carbohydrate demands on the plant for ammonium assimilation depleted the seedlings' reserves, and we probably saw the effects of ammonium toxicity from excessive NH_4^+ availability. The seedlings wilted rapidly even when well watered, which may have been caused by an acid root environment as a result of rapid ammonium uptake, which inhibited absorption of other nutrients and water. The unhealthy seedlings also proved to be a poor environment for bacterial growth, as shown by the fact that severe infections developed only on the healthy seedlings growing on the lowest amount of fertilizer. The use of short-term release Osmocote pellets is therefore not recommended for work done with cabbage seedlings under hot weather conditions.

In conclusion, the use of bioluminescence for nutritional studies has been a valuable tool to trace the pathogen in the same plant for a certain period of time. This new methodology proved that visual symptoms can be misleading in the evaluation of the invasiveness of the pathogen, and do not accurately

reflect the disease severity. Bioluminescence in this pathogen has provided us with a more realistic picture of host-pathogen interactions in black rot of cabbage.

APPENDIX A

Transmission of *Xanthomonas campestris* pv. *campestris* through roots

The many different aspects of the survival and spread of *Xanthomona campestris* pv. *campestris* have been investigated, and it has been determined that the most important source of inoculum for spread throughout cabbage growing areas are the seeds (Cook et al., 1952; Schultz and Gabrielson, 1986). Another source of inoculum is infested debris in cabbage fields under continuous cropping. Studies showed that the pathogen can survive in the soil for 14 to 64 days in a free state, a lower temperature increasing chances of survival; when associated with debris from the host plant, the bacteria remain viable for as long as two years (Schaad and White, 1974; Schultz and Gabrielson, 1986). Papers by earlier researchers indicate the possibility of infection through the roots via wounds, or of the emerging cotyledons (Potter, 1902; Middleton, 1902; Stewart and Harding, 1903; Harding et al., 1904; Drechsler, 1914). Cook and coworkers (1952) poured liquid inoculum of *X. c. campestris* around wounded and unwounded roots of cabbage seedlings and thereby infected 73% of the injured and 13% of the uninjured seedlings. This evidence is quite convincing for root infection.

A second mode of transmission via the root system has been suggested, but is difficult to prove, namely that the bacteria move through the infected plant into the root system and out into the soil to be spread to neighboring plants via soil water or insects (Dr. Alvarez, personal communication).

I decided to try and confirm root transmission with the black rot pathogen 171LIIH-7, tagged with the unique "lux" marker for bioluminescence, by tracing the bacteria from the root system into the leaves, and from the leaves into the roots.

Materials and methods

The genetically engineered *X. c. campestris* 171LIIH-7, has been described elsewhere (Chapter II this thesis). The culture was grown on medium 523 containing 40 ul ml^{-1} of spectinomycin. Before

inoculum preparation, light production was verified. Seeds of *Brassica oleracea* cv. *capitata*, G-C cross, were surface sterilized in 1% Clorox for 10 minutes and germinated in steam sterilized supersoil. After two weeks each of 10 seedlings was carefully transplanted into a 3" pot of supersoil containing infested plant material. The inoculum was prepared in the following way: cabbage leaves with 10-day old black rot lesions caused by 171LIIH-7 were cut into 0.5 cm^2 pieces. It had been determined previously that 1 cm^2 of comparable lesions contains an average of 10^{11} cfu ml^{-1}. The infected pieces were mixed into the potting medium immediately before transplant. The top of the pot around the stem of the seedling was covered with aluminum foil to prevent any splashing of water onto the soil surface. Seedlings received water and nitrogen in the form of ammonium nitrate from the bottom. Two seedlings in clean soil served as controls. Seedlings were grown in the greenhouse and monitored for appearance of symptoms after one week and thereafter. To verify presence of the bioluminescent pathogen in a developing lesion, the seedling was carefully removed and the root system washed free of soil material, covered with plastic wrap, and placed on a piece of X-ray film for a 12 hour exposure.

In a separate experiment, two four-week old seedlings were grown and notch- inoculated as described (Chapter III). Four weeks after inoculation, the infection had progressed from the infected leaf to another leaf, which indicated that systemic invasion had occurred. The roots were carefully washed free of debris, and X-ray film was exposed to the whole seedling for 12 hours. Subsequently, stem sections from the petiole of the infected leaf to the root were surface sterilized, cut into small pieces and placed on 523 agar place containing antibiotics, to confirm the presence of *X. c. campestris* as recorded by X-ray.

Results

Root infection from soil debris. One seedling developed typical black rot symptoms on the first leaf from the petiole up four weeks after inoculation (Fig . 17). X-ray exposure recorded bioluminescence in the root, the petiole and the leaves of another seedling, when the experiment was repeated (Fig. 18) . In each experiment, only one out of the ten replicate plants became infected.

Root infection from leaf tip infection. Leaf blot analysis traced the pathogen from the infection site through the leaf into the petiole only. No light was detected by the film from either the stem or the roots. Laboratory isolation techniques, however, proved that the pathogen had invaded 6 cm into the stem, but had not reached the stem-root interface at the crown of the plant. The same procedure was repeated with another plant, and the same results were obtained.

Discussion

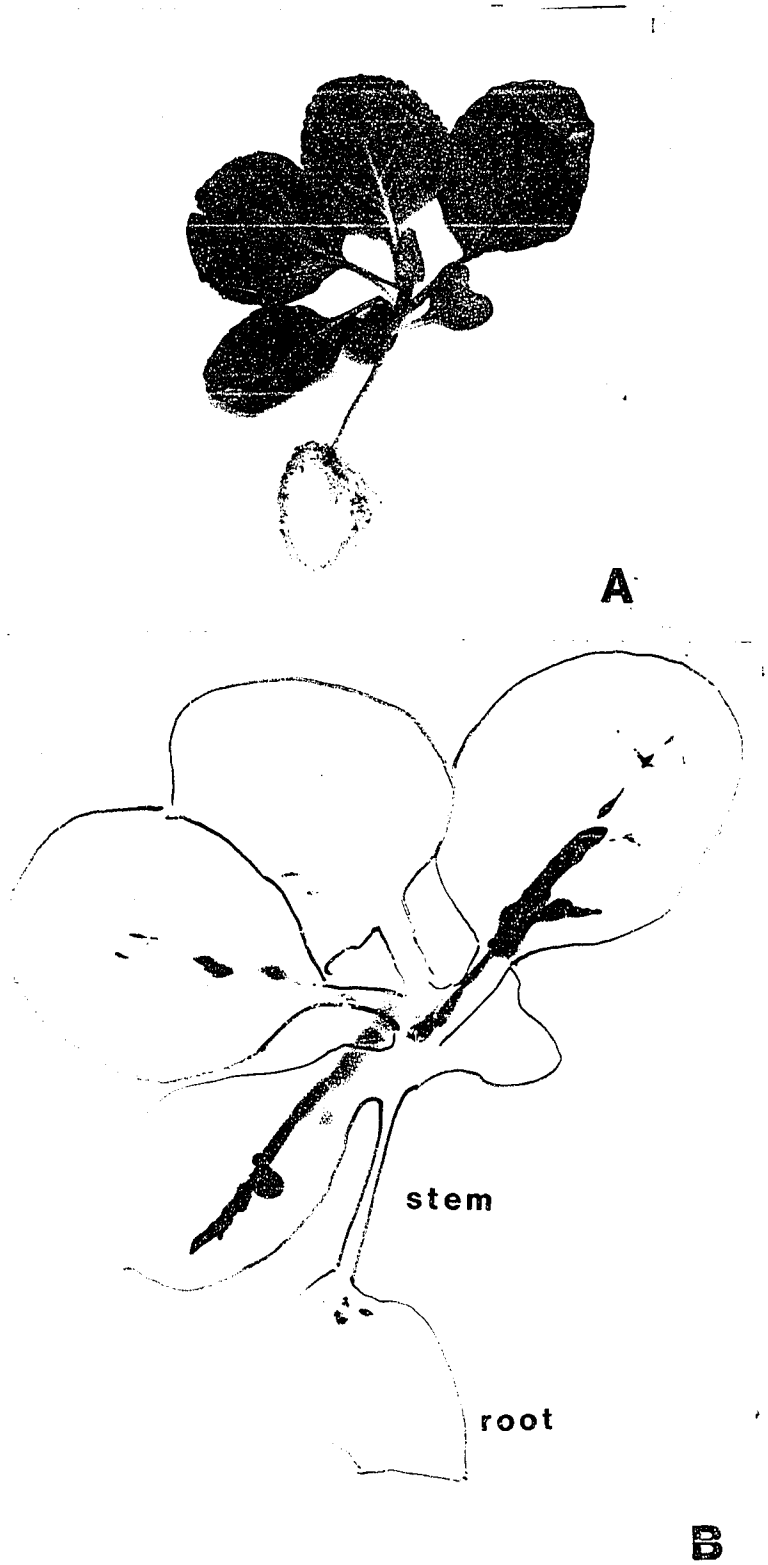
The continuous cropping practice in Hawaii's warm climate often results in epidemics of black rot of cabbage (Alvarez and Cho, 1978). It is believed that infested debris from previous crops represents an important factor in spread of the pathogen. New transplants can become infected by raindrops or irrigation water splashing bacteria from the ground onto the plants, or by wind whipping up dried inoculum on debris (Kuan et al., 1986). Root infection from bacteria in infected soil debris is believed to contribute to the problem.

My experiments add visible proof to the evidence for root infection first reported by Cook et al. (1952). I cannot show whether the pathogen entered the roots directly or through wounds in the roots, but the important fact is that they spread from the soil into the plant; and under natural conditions, wounds are readily made by microorganisms or insects in the rhizosphere. When the experiment was repeated, again only one seedling became infected. This represents a 10% infection rate, which could have serious consequences in the seedbed.

Valid conclusions from the second experiment as to root infection from the plant itself cannot be drawn. At the time of testing, the bacteria had not advanced far enough, and it is difficult to predict the further development of the disease. I have also checked the root system of older cabbage plants with

Fig. 17. Black rot infection of a cabbage seedling through the roots. The lesion is progressing upwards in the petiole of the oldest leaf.

Fig. 18. Black rot infection of a cabbage seedling through the roots. A) Cabbage seedling infected with 171LIIH-7 after growth in soil mixed with infected cabbage debris. Seedling has been rinsed free of soil debris and prepared for bioluminescence recording with X-ray film. B) X-ray exposure pattern of strain 171LIIH-7 in the root area, petiole, and leaves, superimposed over a drawing of the seedling.


A
stem
root
B

severe systemic infections from 171LIIH-7 with X-ray film, but no bacteria were detected. The results of this work do point out the inadequacy of bioluminescence when working with thick (corky) tissues like a stem that will not allow light to pass through. The light path from the root to the bottom of the petiole was blocked by the stem as can be seen in Fig. 18.

In conclusion, bioluminescence does provide us with a unique marker for further soil transmission studies, such as tracing the spread of the pathogen in seedbeds or in soil water.

APPENDIX B

Transmission of *Xanthomonas campestris* pv. *campestris* by whiteflies

Very early investigations into the etiology of black rot of cabbage already indicated that the causative bacterium, *X. c. campestris*, was seedborne, but could also be spread by insects, such as the cabbage butterfly and the harlequin bug, and by insects being covered with bacteria-bearing dust particles moving about the plant (Smith, 1897). Insect punctures were also thought to be responsible for transmission (Russell, 1898). Clayton (1924) went so far as to state that insects appeared to be the major form for dissemination; in his experiments with aphid transmission on cauliflower, all protected plants in cages remained healthy, while unprotected plants became diseased. These findings were subesequently found to be incorrect. Aphids were suspected as secondary agents of infection, but careful experiments could not provide any proof that these insects disseminated the black rot pathogen on cabbage, in spite of the fact that bacteria were isolated from the insects. The researchers concluded that theoretical spread through these insects' feeding and walking was possible, but that laboratory conditions had not been conducive to spread (Brenner, 1904; Stapleton, 1950). Convincing evidence for spread of *X. stewarti* by the corn flea beetle and the toothed flea beetle was presented by researchers who showed that the bacteria overwintered in these insects (Elliott and Poos, 1934). The cabbage flea beetle was thought to have limited potential for transmission of *X. c. campestris* (Shelton and Hunter, 1985), since only 5 out of 35 broccoli plants became infected after beetles, which had fed on diseased plants, were released on them. One hundred percent transmission only occurred when the beetles were sprayed with the bacteria prior to being allowed to feed on the healthy plants.

I have observed during all my work with cabbage seedlings, whether it was done in the greenhouse or even in the laboratory, that plants invariably became infested with whiteflies, and suspected that these insects might have caused some unexplained infections on uninoculated seedlings growing in the same area, however well removed, from infected plants. Whiteflies have been shown to

transmit *X. c. campestris* on geranium (Bugbee and Anderson, 1962). Transmission was also reported on Dolichos bean plants, which became infected with *X. c. phaseoli* through *Bemisia tabaci* , the sweet potato whitefly, at high infestation levels (Sabet et al., 1969).

Since whiteflies thrive under the warm weather conditions in Hawaii, I felt that it was important to establish whether these insects have a major role in the spread of black rot. By using the bioluminescent 171LIIH-7 I can eliminate any doubtful results from possible infections which might come from previously already infected seeds.

Materials and Methods

Brassica oleracea cv *capitata*, G-C cross, seedlings were grown in supersoil in insect-proof cages in a greenhouse under prevailing environmental conditions. Ten four-week old seedlings were inoculated with a suspension of the bioluminescent *X. c. campestris* 171LIIH-7 as described. Three days after inoculation, approximately 100 whiteflies, *Bemisia tabaci* (Gennadius), which had been cultured on healthy cabbage plants, were released on each infected seedling. Duration of feeding was eight days, during which time the black rot infection progressed rapidly. The flies were then blown off the plants into the cage, the plants were removed, and ten healthy seedlings were placed into the cage with the whiteflies. Insects were allowed to feed on the plants for five weeks. Whitefly nymphs were carefully removed from the infected seedlings, crushed in sterile water, and covered with 523 agar containing kanamycin, spectinomycin and chloramphenicol. Plates were incubated at 28 C.

In a second experiment, the experimental design was slightly modified. Seedlings were grown and inoculated as described. Whiteflies grown on clean plants were released onto six infected seedlings inside a large insect-proof cage. Two days later, six healthy seedlings were placed into the same cage, approximately 1.5 foot away from the other plants. Each pot was watered individually by hand through the top of the cage. Great care was taken not to touch the diseased plants. Duration of the experiment was five weeks.

Results and discussion

No *Xanthomonas* was recovered from the whitefly nymphs which had fed on infected cabbage leaves. The agar plates with the nymphs were overgrown with fungi, which interfered with the observation of bacterial colonies, however, light recording with X-ray film would have detected the presence of the bioluminescent bacteria. The film was not exposed.

Black rot lesions did not develop on any of the healthy seedlings, regardless of whether whiteflies, which had previously fed on diseased seedlings, were released on them, or whether they were growing in the vicinity of infected plants supporting a whitefly population. The infection on the inoculated leaves progressed rapidly and became systemic, and the whitefly population rose steadily on both the healthy and infected plants over the 5-weeks of the experimental period. By the end of the period of observation, all the infected seedlings had died from black rot combined with stress from the insects, and the healthy ones exhibited poor growth as a result of the heavy whitefly infestation.

In other studies, which showed transmission of the pathogen by whiteflies, the bacteria were leaf spot pathogens. *X. phaseoli* was found to be transmitted only when very high amounts of whiteflies were present (Sabet, 1969). *Xanthomonas pelargonii*was also recovered from live flies moving around on agar plates, but not from nymphs or dead flies (Bugbee and Anderson, 1962).

The difference in the mode of infection between leaf spot and xylem invading *Xanthomonas* species could explain the negative results in these experiments. Whiteflies feed by inserting their mouthparts into the leaf mesophyll cell and sucking the sap. In the mesophyll infection process, bacteria can easily be injected into a healthy cell by a whitefly which has previously fed on an infected cell and carried the bacteria on the mouthparts. However, for bacteria to gain access to the xylem, an *X. c. campestris* carrying whitefly would have to accidentally walk on a wound with an exposed vascular bundle, or on moisture covering a hydathode, the primary infection site for the black rot pathogen. These are chance events, and may be of importance only at very high whitefly concentrations in

combination with other parameters favoring transmission and infection, such as wind, or other movements contributing to whitefly displacement, moisture to enhance bacterial growth (Kucharek and Strandberg, 1981), or temperatures conducive to the formation of guttation droplets. These factors were absent in the protective environment provided by the cage inside the greenhouse, where no wind disturbed the relatively inactive flies, and temperature fluctuations did not occur.

Ants in combination with whiteflies could represent a problem in a whitefly infested cabbage field. These insects feed on the honeydew secreted by the whiteflies, and could spread bacteria caught in the sticky exudate from leaf to leaf or plant to plant, and into the soil.

The failure of black rot transmission to occur in these experiments cannot be regarded as proof that whiteflies are not agents for the spread of the pathogen. Environmental conditions may have been unfavorable for such a mode of spread, as seemed to be the case in experiments with insects implicated in the spread of *C. sepedonicum* in ring rot of potato (Carter, 1973), where opposing results were obtained from several experiments. I feel that whiteflies are involved in accidental spread as opposed to active spread by insects which are vectors for the pathogens, and that they should be controlled in order to reduce the risk of additional spread of black rot of cabbage by the whiteflies themselves and ants which are attracted by the flies.

APPENDIX C

Considerations in using X-ray film to measure bacterial bioluminescence

1. Plant material. The transmission of light from bacteria growing in plant tissues depends on the composition of the cells surrounding the pathogen. Light emitted from vascular pathogens exposes the film only from vascular tissue in leaves, petioles, and stems of very young seedlings, before corky layers in maturing stems develop and prevent light from passing to the outside.

It is important to have a very tight fit between the infected plant material and the film in order to obtain a clear and accurate leaf blot of the location of the bacteria. Irregularities in the surface of the plant tissue can result in diffusion of light, and a fuzzy X-ray photo.

2. Temperature. Bioluminescence is temperature-dependent. At temperatures of 30 C and above the enzyme luciferase progressively loses its activity. It is therefore recommended to standardize the experimental conditions, and record light production when luciferase has its optimum activity. This can be determined for each transconjugant. When experiments are done under ambient high temperatures, it is essential to move the test material to a permissive temperature for a period of time before bioluminescence can be recorded most efficiently. The duration of the preincubation time depends on the degree and time of exposure to the high temperature and can be experimentally determined.

3. Moisture. Moisture on X-ray film prevents light from exposing the film. Therefore, conditions where the relative humidity becomes saturating should be avoided. Plant parts for light recording should be dry, but slightly moist parts can be wrapped in Saran wrap before attaching the film.

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