The Effect of Copper Sulphate and Host Variety on Angular Leaf Spot (Xanthomonas fragariae) of Strawberry

By

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I dedicate this work to my family, especially my Mother and Father, who have taught me how to wok hard, be the best I can be, and be involved in as many things as possible. Lazy hands make a lazy mind.

I also dedicate this to my new family, my husband Tim Fisher, who has supported me, advised me, and pushed me to accomplish this goal and all my dreams.

I would also like to dedicate this work in the memory of my Uncle Stephen Kennedy who taught me the value of education and is a reason why research is so important. Without research we will never be able to cure the diseases that claim are loved ones at early ages.

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Acknowledgments

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Abstract

Xanthomonas fragariae Kennedy and King (angular leaf spot) is the only known bacterium to infect the cultivated strawberry, *Fragariae x ananassa*. This study investigated host cultivars to determine their susceptibility or resistance levels to the disease. The cultivars Mic Mac and Honeoye were very susceptible to the angular leaf spot disease. Annapolis and Cavendish were moderately susceptible. The cultivar NY113 had low disease ratings in the first growing season and had higher disease ratings the following season. The lowest disease ratings were recorded for Jewel.

The efficacy of copper pesticides at various timings in the Nova Scotia climate were investigated. It was concluded that copper sulphate suppressed the disease but did not completely control it. The best timing of copper sulphate was a repeated application of the compound starting at the bloom stage, followed by four applications at either five or ten day intervals.

Epidemiology studies were also performed to verify the effects of temperature and moisture on the development of the disease. There was some difficulty in the inoculation process of the strawberry plants under artificial environmental conditions.

1. Introduction

Strawberry production is a growing industry, not only in popularity but also economically. In Nova Scotia, strawberries have an approximate farm value of $4 million annually. In 1995, Nova Scotia's 110 strawberry growers harvested 4.5 million quarts from 750 acres, which is a 26% increase from 1994. In Nova Scotia approximately 25% of the crop was u-picked, 67% sold to retail outlets and over 8% of the crop went to processing (Murray, 1996). In 1996, there was another increase in production with 900 acres of land under strawberry production. In Nova Scotia 91% of households buy strawberries during the growing season, while 4% grow their own.

Due to favourable climatic conditions, Nova Scotian strawberry producers have had to deal with many fungal pathogens, but were never concerned with bacterial diseases in the past. In the last five years, Canadian growers have faced an increasing occurrence of a bacterial infection known as angular leaf spot caused by *Xanthomonas fragariae* Kennedy and King. This bacterium is considered the only true bacterial pathogen of the cultivated strawberry, *Fragariae x ananassa*. The bacterium may have been present before, but escaped detection due to the similarity of later stage symptoms to those of common leaf spot (*Mycosphaerella fragariae*) and leaf scorch (*Diplocarpon earliana*). Epstein

(1966) in Wisconsin reported a 75%-80% loss in a 7 ha fruiting field in the cultivar Sparkle. Roberts *et al.* (1996) concluded that disease severity of 25% and 10% in each of two years of that study resulted in 10% yield loss each year among Sweet Charlie plants due to this disease.

Observations in Nova Scotia suggest that the bacterium can overwinter in a field from season to season and spread throughout an entire field. In 1993, the disease was present on three of fifteen northern Nova Scotia strawberry farms monitored; in 1994 it was present on eleven of the fifteen farms. In 1995 ten of eleven farms monitored had the disease. New Brunswick had also reported an increase of angular leaf spot infection in their productive strawberry fields during the 1995 growing season (Kevin McCully, personal communication).

Studies to date on angular leaf spot of strawberry have been merely on the reporting of the presence of infection, the identification of the bacterium, and the proof of its pathogenicity (Hazel 1981). Very little research on the epidemiology of angular leaf spot of strawberry has been reported.

The objectives of this project were to evaluate the efficacy of the copper pesticide, fixed copper, at various timings in the Nova Scotia climate on three strawberry cultivars. The reaction of six host cultivars were also evaluated on their resistance and susceptibility to *X.*

fragariae. The effects of temperature and moisture on disease development were also investigated under controlled environments.

2. Literature Review

Strawberries (genus *Fragaria*) are dicotyledonous angiosperms in the rose family (*Rosaceae*). The strawberry plant may appear to be a stemless perennial herb, but it is neither stemless nor truly herbaceous. The strawberry stem is compressed into a rosetted crown about 2.5 cm long, which is covered on the outside by overlapping leaf bases (stipules). The strawberry crown bears, between each leaf and the crown, axillary meristems or buds. These auxiliary buds expand to form either runners or branch crowns, or also form inflorescences at the terminus of the shoots (Galletta *et al.* 1990).

The runners or stolens, produced from the crown's axillary buds, are two-noded stems. The first runner usually originates from the axil of the first leaf initiated on the crown in the spring. The first internode of the runner elongates and ceases growth with the formation of the "blind" node. This "blind" node grows out another prostrate and elongated branch runner identical to the one from which it arose. A succession of internodes develop at the second node, then leaves and more auxiliary buds form, which produce a daughter plant. The first auxiliary bud of the daughter plant may produce another runner, with two nodes and another daughter plant to form a runner chain. Within a growing season a vigorous plant can expand 10 to 15 runners, with 100

daughter plants rooting from the mother plant (Galletta *et al.* 1990).

The leafless flower stalks (inflorescences) also expand from the crown. The stem supporting each individual flower is called a pedicel. The floral axis of the strawberry flower is swollen at the tip of the pedicel to form a receptacle. In this area hundreds of pistils are found around the receptacle with the ovaries embedded in the receptacle. Outside of and surrounding the receptacle and pistils are a double ring of 20 to 35 stamens, each which consist of a short filament to which the anther is attached. Outside the stamens are usually five separated white petals, which make up the corolla.

In the first growing season the flowers are usually picked in order for the plant's energy to be directed to vegetative growth rather than fruit production. The development of the fruit begins with the mature anthers releasing pollen that fertilize cells within each ovary to form an embryo. This initiates the growth of the embryo into a hard seed with a hard indehiscent fruit called an achene. These dried one-seeded fruits are the true "fruit" of the strawberry. The achene then produces a hormone that causes the receptacle to enlarge and ripen, and forms what is known as the strawberry (Galletta *et al. 1990*).

Strawberry development is closely connected to the prevailing environmental conditions as well. The plant

requires certain temperature, precipitation, light, and air regimes. Unfortunately these environmental conditions at the same time are also contributing to the growth of many pathogens, such as *X. fragariae*.

The angular leaf spot disease is first visible as light green, angular, water-soaked areas on the undersurface of leaves. The angularity of the leaf lesions seem to be due to the restriction of lateral movement of the bacteria through mesophyll tissue by the vascular tissue (veins) and their surrounding external tissues (Preece in Rhodes-Roberts and Skinner 1982). When wet, lesions have a characteristic milky bacterial ooze; when dry, they are covered by a thin clear scaly exudate (Kennedy and King 1962a). Lesions are translucent when viewed with transmitted light but dark green when viewed with reflected light. These spots enlarge and coalesce. The lesions then also appear on the upper surface of the leaf, first as small brown spots, often surrounded by an irregular halo. The yellow halos may be due to the degradation of chloroplasts around lesions, caused by toxins produced by the bacteria (Preece in Rhodes-Roberts and Skinner 1982). The lesions enlarge from 1-4 mm and the tissue becomes dark-brown, necrotic and broken (Rodrigues *et al.* 1978).

In general, bacteria commonly enter plants via stomata and multiply initially in the substomatal chambers to initiate leaf spot disease. Occasionally, infection follows

major veins of the leaf, resulting in veinal watersoaking and spreads extensively from the original point of infection through parenchymatous tissue of the large veins, then to smaller veins and may or may not eventually invade the interveinal portions of the leaf (Kennedy and King 1962a). Characteristically, large numbers of plant pathogenic bacteria are found deeper in the tissues of the intercellular spaces and the xylem vessels. The bacteria move freely in the plant. Preece (in Rhodes-Roberts and Skinner 1982) reported that massive multiplication in the intercellular spaces usually results in necrosis.

Young tissue is much more susceptible than mature tissue and water-soaking greatly increases the chances of infection as it allows free passage of bacteria from outer to inner surfaces (Billing in Rhodes-Roberts and Skinner 1982). Young leaf tissue or leaves on healthy, vigorous plants with adequate fertilizer are more likely to become infected than those on diseased, environmentally stressed, or plants growing where nitrogen, phosphorous, and potassium are deficient (Maas 1984, Kennedy and King 1962b). The active growth phases of plants are the ones during which the greatest spread of bacteria is time-limited because the "hardening" of plant tissues restricts the movement and activity of the pathogen. In addition to the foliar phase of this disease, systemic invasion can occur. Stefani and Mazzucchi (1989) found that *X. fragariae* can move

endophytically along the petioles of infected leaves to reach the plant crowns. In California, vascular collapse (decline) was reported (Hildebrand *et al.* 1967). All cell types of the vascular tissue of the crown may be infected, visually evident is watersoaking at the base of the newly emerging leaves. The bacteria occur intercellularly in vascular tissue, forming pockets in the xylem and cambium that often rupture the tissues of the crown (Kennedy and King 1962a).

Infection of all plant parts, except roots has been observed. The bacterium will blight petioles, runners, and flowers. Fruit adjacent to infected calyxes will also be infected.

Infection of the calyxes appears to be the main concern in the Maritimes. Calyxes develop translucent lesions, similar to foliar lesions. The lesions enlarge and coalesce and eventually the entire calyx of the fruit becomes brown and brittle. The calyxes tend to dry onto the fruit therefore making it difficult to remove the calyx from the berry. This condition along with the poor physical appearance of the fruit makes the fruit unmarketable as either fresh fruit or processed fruit.

X. fragariae is a slow-growing, gram negative, motile bacterium which is 0.4 μm in diameter and 1.3 μm long, with a single polar flagellum (Maas 1984). The bacterium is a nonencapsulated non-sporing rod with round ends (Kennedy and King 1962a). The bacterium has a mucoid nature which is

caused by the production of capsular extracellular anionic heteropolysaccharides, often referred to as xanthans.

The role of extracellular polysaccharides in host-pathogen interactions is not understood. They may play an important role in maintaining the viability of bacterial cells in exudate and plant material under dry conditions (Krieg and Holt 1984). The extracellular polysaccharides may have a role in the persistence of water-soaking in leaves in compatible host pathogen combinations. Extracellular polysaccharides may also have a role in the wilting process in the later stages of infection in vascular collapse diseases by plugging the vessels (El-Banoby and Rudolph 1979, Maas *et al.* 1995).

X. fragariae is strictly aerobic, with an oxidative metabolism of glucose. It does not utilize asparagine as the sole source of carbon and nitrogen. The bacterium utilizes succinate very well, gluconate and tartrate less well, but cannot utilize benzoate and propionate. It can hydrolyse gelatin and is lipolytic. It produces hydrogen sulphide, but not indole, acetone, or urease (Panagopopoulos *et al.* 1978).

Denitrification or nitrate reduction does not occur. *X. fragariae* is chemoorganotrophic, able to use a variety of carbohydrates and salts of organic acids as sole carbon sources (Krieg and Holt 1984).

Small amounts of acid are produced from glucose, mannose, sucrose, and to a lesser extent from arabinose.

Acid is not produced from cellobiose, inositol, lactose, rhamnose, salicine, sorbitol, trehalose, adonitol, dulcitol, and inulin. Acid is not produced in purple milk or litmus milk. These characteristics distinguish *X. fragariae* from the *X. campestris* group (Rodrigues *et al.* 1978).

Strains of the bacterium can tolerate 1% NaCl concentrations, but not 2% or more (Alippi *et al.* 1989). Growth is inhibited by 0.1% triphenyl tetrazolium chloride. Growth factors required usually include the amino acids methionine, glutamic acid, nicotinic acid, or a combination of these. The maximum growth temperature is 32°C. (Mazzucchi *et al.* 1973, Krieg and Holt 1984, Alippi *et al.* 1989).

X. fragariae was originally considered a variant of *X. pruni*, a pathogen that also attacks plants in the Rosaceae. However *X. pruni* differs from *X. fragariae* by the size of cells, lipolytic activity, and most importantly host range. *X. pruni* does not cause angular leaf spot of strawberry (Kennedy and King 1962a). Kennedy and King (1962a) found that a bacteriophage capable of lysing *X.pruni* was unable to lyse *X. fragariae*. Maas *et al.* (1995) however has reported that despite significant efforts at systematic classification of the Xanthomonads, the taxonomic relationship of *X. fragariae* within the genus remains uncertain.

The disease is limited to species and cultivars of strawberry, although two species of cinquefoil *(Potentilla* spp.) and tomato (*Lycopersicon esculentum* Mill.), black

nightshade (*Solanum nigrum* L.) and pepper (*Capsicum annuum* L.) have been artificially inoculated with the bacterium (Maas *et al.* 1995).

The first report of a disease with similar foliar symptoms was made by Linford (1928) in Utah (Hazel 1981). However the disease was first described as angular leaf spot in 1960, in Minnesota (Kennedy and King 1960). It has now been reported in all parts of the USA including Wisconsin (Epstein 1966), California (Hildebrand *et al.* 1967), Kentucky (Powell and Khare 1967), Florida (Howard 1971), and North Carolina (Ritchie *et al.* 1993). It is also present in New Zealand and Australia (Dye and Wilkie 1973), France (Rat 1974), Italy (Surico and Varvaro 1985), Sicily (Mazzucchi *et al.* 1973), Greece (Panagopoulos *et al.* 1978), Brazil (Rodriques *et al.* 1978), and in Argentina (Alippi *et al.* 1989). The widespread distribution of the disease may be by importation of infected plant material, mainly from the USA where environmental conditions are more favourable for survival of the disease. Australia, New Zealand, and Italy can trace the introduction of *X. fragariae* to plants from California (Hazel 1981).

X. fragariae Kennedy and King can be disseminated over short and long distances by runner plants in the market (Calzolari and Mazzucchi 1989). At importation the bacteria are usually dormant on the daughter plants, showing no visual symptoms. This makes it difficult to stop the importation of

the disease into a new region.

Roberts *et al.* (1996) reported that in Florida strawberry plants arriving from the northern nurseries for transplanting in the fall are now frequently infected with angular leaf spot. Maas *et al.* (1995) also reported that infected unrooted runner tips were imported into North Carolina from Canada for rooting as plug plants.

Due to this unintentional international movement of infected plants the European Plant Protection Organization (EPPO) has listed *X. fragariae* as a quarantined pest and has prescribed phytosanitary procedures (Roberts *et al.* 1996). The FAO/IPGRI also lists angular leaf spot as a potential risk in international distribution of strawberry germplasm (Maas *et al.* 1995).

It appears that the production of disease-free plants is essential in controlling this disease. Roberts *et al.* (1996) reported that accurate identification of infected plants is imperative. However the availability of detection techniques are limited. Detection of the disease is difficult because *X. fragariae* grows slowly and can easily be masked by faster growing organisms. Tests are also very time consuming.

A specific indirect enzyme-linked immunosorbent assay (ELISA) was developed to detect *X. fragariae* from symptomatic plant tissue. However it could not detect bacteria on asymptomatic plants (Roberts *et al.* 1996). Therefore current research is being conducted in Polymerase Chain Reaction

(PCR) techniques that is able to detect levels of bacteria in asymptomatic plant tissues by amplification of specific DNA sequences for the disease (Roberts *et al.* 1996; Opgenorth *et al. 1996)*. Opgenorth *et al.* (1996) have generated genomic fingerprints of reference strains of *X. fragariae* using PCR techniques, which are able to accurately and quickly identify *X. fragariae* field isolates on asympomatic plants.

An asset is that *X. fragariae* does not live free in the soil. However it can overwinter on leaf litter or infected crowns. Bacteria were isolated from dried, infected leaves stored 10 years in an herbarium (Hazel 1981).

The bacterium is disseminated through a field by splashing water, such as rain or irrigation. Equipment and people can spread the bacteria throughout the field under moist conditions.

Initiation and development of the disease are favoured by moderate to cool daytime temperatures (around 20°C), low nighttime temperatures (near or below freezing), and high relative humidity. Symptoms seem to diminish with the onset of hot weather. The number of leaf lesions per leaf was markedly reduced at 26°C regardless of humidity conditions (Kennedy and King 1962b). In Maryland, the strawberry is apt to become severely infected by *X. fragariae* in August (Hazel 1981).

Long periods of precipitation, sprinkler irrigation used to protect plants from freezing, and heavy dews in the spring

also favour the disease (Hazel 1981). Dew allows for dissemination of bacteria exuding from early-forming lesions. Bacteria suspended in dew, can then gain access to the substomatal cavities and the intercellular spaces upon stomatal opening (Hazel 1981). This could be passive entry due to random movement of bacteria into the stomata or due to a positive chemotaxis induced by certain dissolved compounds originating from the intercellular spaces. This theory has never been investigated (Hazel 1981).

It is evident that effective control measures must be obtained. Control of this disease is difficult. The best control is achieved using the antibiotics streptomycin and streptomycin + oxytetracycline (Alippi *et al.* 1989), but these antibiotics are unavailable for plant therapeutic use in Canada. The use of antibiotics is prohibited because of the potential transfer of drug resistance from bacteria associated with plants to those associated with animals and humans (Byrde 1979 in Rhodes-Roberts and Skinner 1982). Copper compounds give some control but repeated applications at intervals of seven days or less may be phytotoxic (Hazel 1981).

Marco and Stall (1983) stated that the relatively low cost and low toxicity to mammals of fixed copper compounds give them the advantage over other chemicals for control of foliar bacterial diseases. However, very little is known about the activity of copper as a bactericide, even though

much attention has been given to fixed copper as a fungicide. It was suggested by Marco and Stall (1983) that the copper merely hardens the plant surface, resulting in less penetration by bacterial pathogens. Marco and Stall (1983) also stated that the efficacy of fixed copper compounds in control of bacterial diseases has been variable. There is evidence of resistance to the copper compounds developing among certain strains of bacterial pathogens and plants.

The limited effectiveness of copper sulphate on this disease may be accounted for by the fact that the Cu^{2+} ion binds with organic substances available on the plant surface rather than affecting the bacteria. Copper can form soluble complexes with amino acids, hydroxyacids, and carbohydrates. A large number of organic substances have been described in leachates from leaves, including simple sugars, organic and amino acids, phenolics, and vitamins (Menkissoglu and Lindow 1991 and Goto *et al.* 1993).

To increase the effectiveness of copper Lee *et al.* (1993) suggested adding iron to the copper bactericide. In the presence of iron, less free copper ions were needed to inhibit the growth of bacteria. The iron competes for copper binding sites in copper binding proteins. Thus in the presence of iron, copper is no longer bound and can enter the plant's cytoplasm and affect the bacteria.

3. Materials and Methods

3.1 Bacterial Sample Collection

Infected leaf samples were collected from various productive strawberry fields throughout north-central Nova Scotia, and from a nursery farm which had plants imported from Florida. Infected leaves were refrigerated in plastic bags until isolation of the culture could be performed.

3.2 Isolation of Bacterial Culture

Leaf samples containing both translucent lesions and healthy leaf tissue were cut into small sections (approximately 2 cm x 2 cm) with a sterile knife and surface sterilized in 0.6% sodium hypochlorite for 10 seconds. The leaf sections were then rinsed in distilled water and macerated in 5 mL of distiled water with a mortar and pestle. A sterile wire loop was dipped into the bacterial suspension and was streaked onto a solid agar plate. The wire loop was streaked back and forth 3-5 times to form lines on the agar plate. The wire loop was then re-sterilized under a flame. The media plate was turned 90^0 and the wire loop was pulled through the previous lines and streaked 3-5 more lines. The media plate was turned 90^0 again and the enflamed loop steak 3-5 more lines. This procedure ensured that the bacterial suspension was covering the entire media plate, but also by the continued streaking through the lines would cause the

suspension to become diluted to the point where separate colonies of the bacterium would be produced. The separate colonies would ensure that a pure culture of the bacterium would be used throughout the remaining experiments (Kiraly *et al.* 1974). The media plates were incubated at 22°C until bacterial growth was apparent.

Three different media were tested to determine which one was the best for the isolation of *X. fragariae*. The media were also tested to see which one was able to maintain the bacterium's pathogenicity over a long period of time (approximately for six months).

The tested media were the following:

YDC:

Yeast extract-dextrose-$CaCO_3$ (YDC) was prepared by dissolving 10 g yeast, 15 g agar, and 20 g $CaCO_3$ in 800 mL double distilled water, and autoclaving the solution before mixing it with 200 mL autoclaved 10% dextrose solution.

This is a differential medium for *Xanthomonas* species (Dhingra and Sinclair 1985).

SX

SX agar was prepared by dissolving 10 g soluble potato starch, 1 g beef extract, 5 g ammonium chloride, 2 g dipotassium phosphate, 1 mL methyl violet 2B, 2 mL methyl green, and 15 g agar in 1000 mL of distilled water. The

solution was autoclaved and mixed with 5 mL autoclaved cycloheximide.

SX medium is used for the isolation of *Xanthomonas*. It selectively depends on starch utilization. The methyl green and methyl violet B are added to increase selectivity and improve colony differentiation (Dhingra and Sinclair 1985).

SPA

Sucrose peptone agar (SPA) was prepared by dissolving 20 g sucrose, 5 g peptone, 0.5 g K_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, and 15 g agar in 1000 mL of distilled water. The solution was autoclaved.

SPA medium is a non-selective medium for *X. fragariae*. It can be used to maintain pathogenicity of the culture (Dhingra and Sinclair 1985).

3.3 Strawberry Plant Material

Certified disease free strawberry cultivars Honeoye, Jewel, Mic Mac, Cavendish, and Annapolis were obtained from Keddy's Nursery, Lakeville, Nova Scotia. The cultivar NY113 was obtained from Michel Lareau, Horticultural Development and Research Centre, Agricultural Canada, St-Jean-sur-Richelieu, Quebec, Canada. The plants were maintained in the packing boxes in a cold room until they were planted.

3.4 Inoculum Preparation and Standardization

In order for the experiment to be repeated in future studies, the inoculum concentration used had to be standardized. A dilution series was used to quantify the concentration of bacteria present in the inoculum used. The dilution series (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) was plated onto nutrient agar plates (3 g beef extract, 2.5 g glucose, 5 g peptone, 15 g agar, and 1 L water) and colonies were quantified using a Quebec Colony Counter. A spectrophotometer at 520 nm was used to read the absorbance of the dilutions. A curve of CFU/mL versus absorbance readings was then prepared. The concentration of inoculum could then be standardized by simply referring to this standard curve based on the suspension's absorbance reading (Figure 1).

3.5 Inoculation Procedure

The optimum method of inoculation had to be determined for this pathogen, so two techniques were tested: spray inoculation and pin prick inoculation.

One loopful of *X. fragariae* culture grown on YDC medium for 8 days was placed into 50 mL of Difco nutrient broth. The flask was shaken at 24°C for 24 hours.

Six plants of the susceptible cultivar Honeoye were planted in Promix-BX and maintained in a greenhouse for five days before inoculation was performed. Two plants were

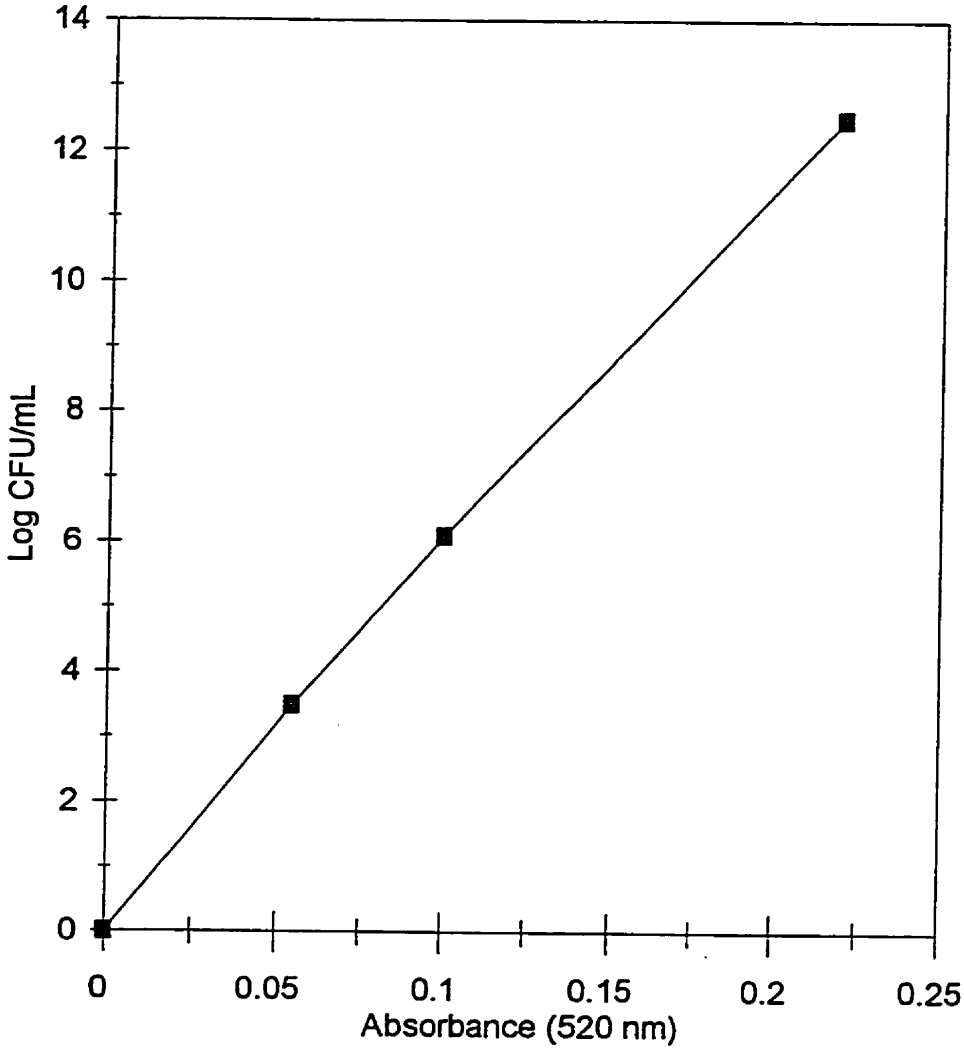

Figure 1: Standard curve for absorbance versus CFU/mL for bacterial inoculum suspensions.

soaked with undiluted inoculum using an atomizer sprayer. Two plants were inoculated with inoculum diluted with equal parts of distilled water. Two other plants were inoculated with inoculum diluted with two parts distilled water.

Stems and veins on each plant were also pricked with a sterilized needle to see if bacterial infection would be enhanced through a wound. The pricked area was marked with a piece of string.

Plants were placed on a mist bench that misted automatically every half hour. Plastic bags were placed over each plant after inoculation to increase the humidity level. After 72 hours the plants were removed from the mist bench and placed in a greenhouse until symptoms were visible.

3.6 Pathogenicity Testing

Once an inoculation method was identified, then the presence and identification of the pathogen had to be determined. Four strawberry plants (cv. Honeoye) were planted in Promix-BX and maintained in a greenhouse for five days. A loopful of *X. fragariae*, grown on YDC medium for five days, was transferred into 500 mL of Difco nutrient broth and shaken at 24°C for 7 days.

Two healthy Honeoye plants were placed in the dark for 12 hours and then were inoculated with the bacterial suspension using an atomizer sprayer. Leaves were sprayed until they were soaked (approximately 5 mL).

Inoculated plants were covered with plastic bags and placed on a mist bench for two days. Plants were then placed in a greenhouse until symptoms were visible.

Once symptoms were visible (approximately after 12 days), infected leaf sections were surface sterilized with 0.6% sodium hypochlorite for 10 seconds. The leaf sections were then plated onto YDC medium and incubated at 22°C to verify that *X. fragariae* was the actual pathogen causing the infection.

3.7 Host Cultivar Field Trial

A 20.6 m x 25 m area of land was harrowed for the host cultivar trial. Six cultivars of strawberry were planted on 6 June 1995. Honeoye and Mic Mac were selected as susceptible cultivars. The cultivars Annapolis and Cavendish were selected as the two most popular cultivars in Nova Scotia. The two remaining cultivars Jewel and NY113 were chosen because they reportedly have some resistance to *X. fragariae*.

Treatment 1 = Mic Mac

Treatment 2 = Honeoye

Treatment 3 = Cavendish

Treatment 4 = Annapolis

Treatment 5 = Jewel

Treatment 6 = NY113

Each plot consisted of three rows of strawberry plants, with 10 plants in each row. There were 40 cm between plants within a row, and 100 cm between rows. Each plot was replicated 4 times. The trial was set up as a randomized complete block design. The fifth plant in the middle row of each plot was a Honeoye plant infected with *X. fragariae*.

Twenty-four Honeoye plants were planted in Promix-BX in 26 cm x 52 cm plastic trays (12 plants/tray). They were grown in a greenhouse for five days.

Eight flasks of 200 mL Difco nutrient broth were inoculated with *X. fragariae* grown on YDC medium for 7 days. The flasks were shaken at 24°C for five days.

After seven days, the plants were inoculated using an atomizer sprayer with the *X. fragariae* suspension having a concentration of 1.46×10^{10}.

Each tray of plants was placed in a large plastic bag and placed on the mist bench (25.5°C). After 4 days the bags were removed and the trays were placed in the greenhouse (26°C) until they could be planted into the field plots.

The trial was monitored for spread of bacterial infection. Incidence and severity data were recorded periodically, mainly on a 3-4 week basis. Each plant was given an overall rating from 0 - 20, where 0 was no symptoms present, and 20 was total necrosis of the plant. The rating scale was a modification of the disease severity rating scale used in Maas (1984). The rating scale used in this study

used a larger range of rating values to account for the differences in disease development. Refer to Table 1 for the disease severity rating scale. The individual ratings for the nine plants in the middle row in each treatment plot, excluding the inoculum source plant, were averaged for each plot. The trial was rototilled and hoed regularly to control weed populations. The trial was studied over the 1995 and 1996 growing seasons. Winter survival of the bacterium was also evaluated in the spring of 1996 by recording any sign of lesions on the leaves.

3.8 Statistical Analysis of Host Cultivar Trial

The analysis was based on a randomized complete block design. Each plant was considered the experimental unit. The treatments were randomized within the blocks. The disease rating data were analyzed in the SAS program using a repeated measures procedure. The statistical model was:

$$Y_{ij} = \mu + \tau_i + \beta_j + \epsilon_{ij}$$

where: μ = overall mean

τ = treatment (cultivar) effect

β = block effect

ϵ = NID(0, σ^2) random error term

$i = 1,2...6$

$j = 1,2...4$

Table 1. Disease severity rating scale.

Rating Number	% leaf surfaces covered with lesions
0	No sign of any lesions present
2	10 % coverage, pin point lesions
4	20% coverage
6	30% coverage
8	40% coverage
10	50% coverage, water-soaking is apparent
12	60% coverage, lesions beginning to coalesce
14	70% coverage, some mucus present
16	80% coverage, mucus covering lesions
18	90-100% coverage, leaves becoming necrotic.
20	Total necrosis of leaves due to disease.

On the first two observation dates, week 3 and week 11, little infection was recorded for the cultivar treatments. Therefore these dates were excluded from the statistical analysis. The repeated measures analysis considered the data from week 16 as a control and were contrasted with the other observation dates.

The assumptions of normality and constant variance of the data were checked initially in the statistical analysis. The assumptions were valid for weeks 57, 62, and 68, however the assumption of normality for week 16 was not met. Usually transformations to the data would have been performed in order to make the data normal. In this case most of the ratings for week 16 were 0, therefore transformations would not have affected the results.

Throughout the analysis there was no significant effect on the data due to the blocking factor at an α level of 0.05. So the blocking factor was not accounted for in the remainder of the analysis.

The test for sphericity (independence) was performed and it was calculated that sphericity was violated ($P \leq 0.0094$). Thus there was no independence among the data. Due to this violation, multivariate or MANOVA analysis was performed. From this analysis it was concluded that the Wilks' Lambda coefficient for the date of observation had a significant ($P \leq 0.0001$) effect on the results. Therefore the disease rating means were confirmed to be uniformly changing over

time.

3.9 Copper Sulphate Trial

A 70 m x 25 m area of land was harrowed and prepared for the copper sulphate trial. Three cultivars of strawberry, Honeoye, Jewel, and Cavendish were planted on 7 June 1995. Each plot consisted of 3 rows of strawberry plants, with 10 plants in each row. There were 40 cm between plants within a row, and 100 cm between rows. Each plot was replicated 4 times. The trial was set up as a 5 x 3 factorial design. The fifth plant in the middle row of each plot was a Honeoye plant inoculated with *X. fragariae* as in Section 3.7.

The trial had fifteen treatments, where each of the three cultivars (cultivar 1 = Honeoye, cultivar 2 = Cavendish, and cultivar 3 = Jewel) was subjected to a copper sulphate application at various timings. The four timings were 1. pre-bloom, 2. full bloom, 3. full bloom and repeated every 5 days (up to 4 times), and 4. full bloom repeated every 10 days (up to 4 times). The fifth treatment was a control, where no copper sulphate was applied.

Copper sulphate was sprayed at 2.5 kg/ha in 1000 L of water, the lowest recommended rate. A CO_2 sprayer was used at 60 PSI. The copper sulphate was applied in the two growing seasons.

Disease incidence and severity were recorded periodically. Each plant was given an overall rating from 0

- 20, where 0 was no symptoms present, and 20 was total necrosis of the plant as outlined in Table 1. The individual ratings for the nine plants in the middle row in each treatment plot, excluding the inoculum source plant, were averaged for each plot. The trial was rototilled and hoed regularly to control weed populations. The trial was conducted over the 1995 and 1996 growing season. In the spring of 1996, the trials were evaluated to see if any of the treatments had eliminated the bacterium over winter. Plots were also evaluated for any winter damage attributable to the copper treatments.

3.10 Statistical Analysis of the Copper Sulphate Trial

This experiment was a 5 x 3 factorial design with three cultivars at 5 levels of spray timing. The disease rating data were analyzed in the SAS program using a repeated measures procedure. The statistical model for this design was:

$$Y_{ijk} = \mu + \tau_i + \beta_j + \tau\beta_{(ij)} + \epsilon_{ijk}$$

where: μ = overall mean

τ = cultivar effect

β = spray timing effect

$\tau\beta$ = interaction effect of variety and spray timing

ϵ = NID(0, σ^2) random error term

i = 1,2,3

j = 1,2...5

k = 1,2,3,4 # of reps

On the first two observation dates, week 3 and week 11, only slight infection was observed. Therefore these two dates were excluded from the statistical analysis. The analysis began with the ratings recorded on week 16 which was considered as a control and was contrasted with the other observation dates. Since week 16 was used in the analysis as a control in the contrast procedure, the analysis basically contrasted the 1995 growing season with the 1996 growing season's dates of observation.

Initially the assumptions of normality and constant variance of the statistical model were validated. The assumption of normality was not valid for the weeks 57, 62, or 68 due to the fact that there were many zero values in the data sets which caused non-normality to occur. However no transformations could be performed to alleviate this problem.

The test for sphericity (independence) ($P \leq 0.0000$) was performed and it was calculated that sphericity was violated. Thus there was no independence among the data. Due to this violation, multivariate or MANOVA analysis was performed. From this analysis it was concluded that the Wilks' Lambda coefficient for the date of observation had a significant ($P \leq 0.0087$) effect on the results. Therefore the disease

rating means were confirmed to be changing uniformly over time.

3.11 Growth Chamber Studies

There was some concern that using nutrient broth as a liquid inoculum source would affect the natural infection pathway of the bacterium. Therefore a study was set up to determine the effect of nutrient broth on the inoculation period.

Twenty-four Honeoye strawberry plants were individually planted in Promix-BX in 20 cm plastic pots. They were grown for seven days in a growth chamber operating at 24°C, high relative humidity and a 14.5 h photoperiod (98 $\mu Es^{-1}m^{-2)}$ provided by fluorescent and incandescent lights to mimic natural daylight conditions. The plants were subjected to 4-5 hours of darkness before inoculation. The dark period before inoculation ensured that the stomata would close in the dark and then tend to open as soon as a light source was provided.

A loopful of *X. fragariae,* previously cultured on YDC medium for five days, was placed in 200 mL of nutrient broth, and placed on a shaker for 48 hours. Dilutions of sterile water to noninoculated nutrient broth (N. B.) were prepared (Table 2).

Table 2:Dilution series for growth chamber study

%Nutrient Broth	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
Sterile H_2O (mL)	10	9	8	7	6	5	4	3	2	1	0
N.B. (mL)	0	1	2	3	4	5	6	7	8	9	10

Then 100 μL of the bacterial suspensions were put into each individual dilution flask. Approximately 3 mL of each dilution was sprayed onto each of two strawberry plants using an atomizer sprayer. There were two Honeoye plants used for each dilution treatment.

After inoculation, plants were returned to the growth chamber for a two week period. Symptoms were recorded periodically.

3.12 Temperature Study

Strawberry cultivars Honeoye and Jewel were used. Honeoye was used since it is considered a susceptible cultivar to this bacterium, while Jewel is considered to be tolerant to the disease. Sixteen plants of each of the two cultivars were individually planted in Promix-BX in 20 cm plastic pots. Growth chambers were set at a high relative humidity (90%) and there were 14.5 hours of daylength at 98 $\mu E\ s^{-1}\ m^{-2}$ using fluorescent and incandescent lights.

The experiment was conducted at four different temperature settings: 16°C, 20°C, 23.5°C, and 27°C. Optimum bacterial growth is believed to occur around 20°C - 23°C, so

extremes in both directions (16°C and 27°C) might limit growth. Four plants of each of the two cultivars were used for each treatment.

The plants were grown in the growth chamber for two days and subjected to six hours of darkness before inoculation to ensure that the stomata would close in the dark and tend to open once a light source was provided.

X. fragariae was grown on YDC medium for six days. These colonies were transferred to nutrient broth and shaken for 48 hours. The solution was then diluted in three parts sterile distilled water to produce a concentration of $1.15x10^{10}$ CFU/mL.

The plants were sprayed with 3 mL of the inoculated nutrient broth using an atomizer sprayer. Each plant was placed in a large plastic bag to maintain high humidity. Plants were then placed in the growth chambers for symptoms to develop. Four plants of each cultivar were used for each temperature setting.

An infected leaf from each cultivar was removed every 24 hours for 168 hours and surface sterilized with a 10% javex solution for 20 seconds. The leaves were cut into 2 x 2 cm sections and were washed in sterile water. Leaf samples were then ground into a slurry using a sterile mortar and pestle. The slurry was allowed to sit for 30 seconds, so that the bacteria could exude from the leaf tissue cells. Aseptic techniques were used to transfer the leaf slurry into a test

tube of 2 mL of sterile water Aseptically, 0.1 mL of each suspension was plated onto nutrient agar plates using a micropipeter, and spread evenly with a sterile glass rod. The inoculated plates were incubated at 22°C for 48 hours, and the number of bacterial colonies per cm^2 of leaf tissue was counted using a Quebec Colony Counter. The experiment was replicated.

3.13 Statistical Analysis for temperature Study

The data for the bacterial counts for the various temperature settings were statistically analyzed using repeated measures analysis in SAS. The statistical model was:

$$Y_{ij} = \mu + \tau_i + \beta_j + \epsilon_{ij}$$

where: μ = overall mean

τ = temperature effect

β = cultivar effect

ϵ = NID(0, σ^2) random error term

i = 1,2,3,4 # of temperatures

j = 1,2,3,4 # of reps

3.15 Moisture Study

Strawberry cultivars Honeoye and Jewel were used. Honeoye was used since it is considered a susceptible cultivar to this bacterium, while Jewel is considered to be

tolerant cultivar to the disease. Sixteen plants of each of the two cultivars were individually planted in Promix-BX in 20 cm plastic pots. Growth chambers were maintained at 14.5 hours of daylength at 98 $\mu E\ s^{-1}\ m^{-2}$ using both fluorescent and incandescent lights. The plants were grown in the growth chambers for two days and subjected to six hours of darkness before inoculation to ensure that the stomata would open once a light source was provided. The plants were then placed in a dew chamber for six hours before inoculation to moisten the leaves. The dew chamber was set at the following parameters: wall temperature 25°C, and water temperature which provided humidity to ambient air was 45°C. The cooler wall temperature causes condensation (dew) to form on the plant surfaces.

X. fragariae was grown on prepared YDC medium for seven days. These colonies were transferred to nutrient broth and shaken for 48 hours. The solution was then diluted in three parts sterile distilled water to obtain a concentration of $1.58x10^9$ CFU/mL.

The plants were sprayed with 3 mL of the inoculated nutrient broth using a sterile syringe. Inoculated plants were placed in the dew chamber for 0, 1, 3, or 6 hours. There were four treatments with each having four plants as replications.

Upon removal from the dew chamber excess moisture was dried using a hot air dryer. The plants were then returned

to a growth chamber operating at 20°C.

The number and size of lesions on each plant were recorded each day for 10 days to verify if the duration of moisture had an effect on the development of the disease.

3.15 Statistical Analysis for Moisture Study

The data for the various moisture durations were statistically analyzed using a standard GLM procedure in SAS. The statistical model was the following:

$$Y_{ij} = \mu + \tau_i + \beta_j + \epsilon_{ij}$$

where:

μ = overall mean

τ = moisture effect

β = cultivar effect

ϵ = NID(0, σ^2) random error term

i = 1,2,3,4 # of moisture duration levels

j = 1,2,3,4 # of reps.

4. Results

4.1 Isolation of Bacterial Culture

The YDC medium was the best medium to isolate and maintain the growth of *X. fragariae* on a short term basis (< 4-6 months). The bacterium produced characteristic circular (2-5 mm) bright egg yolk colored colonies on the medium within 3-5 days.

The SX agar was not a good medium for the isolation of *X. fragariae*. Distinct colonies were never visible. The mucoid nature of the bacterium was still evident, however it was difficult to separate single colonies of the bacterium.

As in terms of storage the YDC medium was not good at maintaining the pathogenicity of the bacterium. The SPA medium, however, was able to support growth of the bacterium and maintain the bacterium's pathogenic properties over a longer period of time (6 months-14 months). The culture produced circular mucoid colonies on this medium, however the colonies were a pale whitish yellow color, similar to the colony color of *Pseudomonas* on this medium, making it difficult to distinguish the two species.

4.2 Inoculation Method

The spray inoculation method achieved the best disease levels. The atomizer sprayer provided a uniform application of the bacterial inoculum with enough pressure to apply the

bacterium to the plant's leaf surfaces. Applying the inoculum to the point of run-off was necessary to induce infection; applying less inoculum did not produce disease symptoms.

Various inoculum densities were used in the initial inoculation procedure, though the density was not initially quantified. However the best results were achieved with the 1:2 ratio (50 mL inoculum:100 mL distilled water). Small minute pinpoint lesions were evident on the undersurface of the leaves within 72 hours in the mist chamber. No visual symptoms developed on the plants sprayed with full inoculum or the 1:1 inoculum dilution.

Disease development was only visible when the infected plants were maintained under the plastic bags on the mist bench. Once the plants were removed from the mist bench to the greenhouse, symptoms tended to diminish and further disease failed to develop.

The pinprick method of inoculation did not produce natural angular leaf spot symptoms. The needle wound on the strawberry plant stems resulted in small brown necrotic areas, which eventually dried and caused the stem to split and break the epidermis of the stem tissue. The pinprick method on the leaves created better results; circular, angular lesions formed on the leaf surface. However the lesions never produced the water soaked appearance characteristic of angular leaf spot disease. The lesions

were brown and necrotic and the tissue around the infected wound became dry and necrotic.

Spraying the bacterial suspension on the undersurface of the leaves resulted in more lesions and water-soaking of the leaf tissue to develop, compared to spraying the suspension on the upper surface of the leaves.

4.3 Identification of Bacterium

The inoculation of the strawberry plants was more successful when the plants were at a younger growth stage, the optimum age being two weeks old. Plants inoculated when older than two weeks did not develop the typical water soaked lesions on the leaves.

Koch's postulates were performed. The inoculated Honeoye plants developed yellow minute lesions on the leaves similar to initial natural angular leaf spot symptoms. When the infected leaf samples were plated onto YDC medium the characteristic bright yellow circular colonies developed. Based on this presumptive growth characteristic, it was concluded that the bacterium was *X. fragariae*.

4.4 Field Trials

After four days under the plastic bags on the mist bench, minute (1mm) water soaked areas started to develop on the undersurface of the Honeoye leaves. Once the plants were removed from the mist bench and placed in the greenhouse,

symptoms tended to diminish. However there were initial signs of water congestion in the leaf tissue and minute pin point lesions developing on the upper surface of the inoculated leaves.

The location of the field trials was ideal for the requirements of a strawberry crop. The soil pH was approximately 5.4.

After 12-13 days from being planted in the field plots, some of the inoculum source plants within the field plots started to wilt and appeared somewhat stunted probably due to shock of high temperatures and lack of initial soil moisture. These were replaced by freshly inoculated plants. When the new inoculum source plants were transplanted, it was a cloudy day and there was adequate moisture in the soil due to a rainfall several days earlier. These plants did survive and and were able to transmit the disease to other plants in the trials.

4.5 Host Cultivar Trial

The repeated measures analysis indicated a significant ($P \leq 0.0172$) difference in disease severity from week 16 to week 57. There was also a significant ($P \leq 0.004$) increase between the disease rating means from week 62 and week 68. Due to the first two observation dates being excluded from the statistical analysis, week 16 is the only date used from the 1995 growing season. Since this date was used in the

analysis as a control in the contrast procedure, the analysis basically contrasted the 1995 growing season with the 1996 growing season's dates of observation. The statistical analysis indicated a significant ($P \leq 0.0837$) interaction between the observation dates and the disease severity ratings at a 90% confidence interval, however it was not significant at a 95% confidence interval. There was a highly significant ($P \leq 0.0001$) increase observed in disease severity between week 16 and week 62, and between week 16 and week 68 ($P \leq 0.0001$). The repeated measures analysis also indicated a highly significant ($P \leq 0.0008$) increase between the ratings taken at week 62 and week 68.

At each observation week there were significant differences observed among the six cultivars studied. There was no significant difference initially observed at week 16, however as time progressed there was a more apparent difference observed (Table 3). This change in disease development and significant difference among the varieties was compared to the environmental conditions present at the time of the observations. However no correlation could be found between temperature or precipitation and the increased disease severity. There was very little difference between the two growing seasons. The average temperature for the 1995 growing season (May - September) was 14.7°C and the total amount of rainfall was 540.3 mm. The 1996 growing season's average temperature was 14.8°C and the total

Table 3: Mean disease ratings* for the six strawberry cultivars in the host cultivar field trial.

	1995	1996			
Cultivar	Week 16	Week 57	Week 62	Week 68	Mean for cultivar (1996 only)
Mic Mac	2.193[a]	6.330[a]	8.943[a]	10.808[a]	8.693
Honeoye	0.165[a]	1.585[b]	6.110[ab]	10.058[a]	5.918
Cavendish	0.168[a]	1.223[b]	2.695[bc]	4.025[bc]	2.647
Annapolis	1.163[a]	0.915[b]	4.473[abc]	3.750[bc]	3.046
Jewel	0.445[a]	0.028[b]	0.000[c]	1.000[c]	0.343
NY113	0.055[a]	2.085[b]	4.085[bc]	8.168[ab]	4.779
Mean for obs. Dates	0.698	2.028	4.384	6.302	
LSD	2.312	2.197	4.598	4.810	

Values followed by the same letter within columns are not significantly different according to the LSD listed at $\alpha \leq 0.05$.

* Disease severity ratings are based on a scale that 0 = no lesions present and 20 = total necrosis of the leaves due to the disease.

rainfall was 647.9 mm.

Overall, Mic Mac had the highest mean disease rating. At week 68 Mic Mac had a disease severity rating of 10.808. Honeoye had a relatively low rating of 0.165 at week 16, however increased throughout the season and had a disease severity rating of 10.058 at week 68. At week 16 the NY113 cultivar had the lowest mean disease rating of 0.055, however as time progressed the disease rating also increased. At week 68, NY113 had a disease severity rating of 8.168. Jewel maintained the lowest disease rating throughout the study, with a disease severity rating of 0.028 at week 57 and 1.000 at week 68 (Table 3). Annapolis and Cavendish maintained moderate levels of disease severity ratings throughout the study.

4.6 Copper Sulphate Trial

The general linear models procedure in the repeated measures analysis detected significant ($P \leq 0.0089$) differences among treatments at week 16 (Table 4). There was no significant difference among the control, prebloom, and full bloom copper sulphate sprays, along the repeated application of copper sulphate every ten days. However there was a significant difference among all copper sulphate application timings and the control. There was no significant ($P \leq 0.5131$) difference among the treatments at week 57.

Table 4: Effect of copper sulphate treatments on disease severity ratings *.

Treatments	1995	1996			
	Week 16	Week 57	Week 62	Week 68	Mean for treatments (1996 only)
1	2.416[ab]	1.182[a]	1.701[ab]	1.173[ab]	1.352
2	1.748[ab]	0.787[a]	0.667[b]	1.380[ab]	0.945
3	0.491[b]	0.259[a]	0.018[b]	0.278[b]	0.185
4	1.028[ab]	0.500[a]	0.000[b]	0.556[b]	0.352
5	3.648[a]	0.695[a]	3.453[a]	3.592[a]	1.544
Mean for obs. Dates	1.866	0.685	1.168	1.396	
LSD	2.832	1.141	2.276	2.610	

Values followed by the same letter within columns are not significantly different according to the LSD value listed at $\alpha \leq 0.05$.

* Disease severity ratings are based on a scale that a rating 0 = no lesions present and 20 = total necrosis of the leaves due to the disease.

The statistical analysis did indicate a highly significant ($P \leq 0.0001$ and $P \leq 0.0289$) difference among the copper sulphate treatments at week 62 and at week 68 respectively. However at week 62, there was no significant difference among the control and the prebloom spray, and at week 68, there was no significant difference among the control, prebloom spray, and the full bloom spray.

The cultivars responded differently to the copper sulphate treatments throughout the two year study as well. Overall in the Honeoye the disease ratings were significantly ($P \leq 0.250$) higher in the control plots. This indicates that the application of copper sulphate did suppress the disease in Honeoye. There was no significant difference between the other treatments, however treatment 1, which was the prebloom spray and treatment 2, which was one application of copper sulphate, had higher levels of disease compared to treatment 3 and treatment 4, which are repeated applications of the copper sulphate compound (Table 5).

There were also high disease ratings in the control plots of the Cavendish cultivar. However in this cultivar there was no significant ($P \leq 0.1994$) difference between the control and treatment 1, the prebloom spray. There was also no significant difference among the other treatments. However treatment 3, the repeated copper sulphate application every five days, had the numerically lowest disease severity rating (Table 5).

Table 5: Effect of copper sulphate treatments on overall disease ratings* for three cultivars.

Treatments	Honeoye	Cavendish	Jewel	Mean for treatments
1	1.551[b]	1.194[ab]	0.389[a]	1.045
2	1.305[b]	0.768[b]	0.278[a]	0.784
3	0.227[b]	0.260[b]	0.046[a]	0.178
4	0.222[b]	0.824[b]	0.005[a]	0.350
5	3.246[a]	2.148[a]	0.375[a]	1.923
Mean for cultivars	1.310	1.039	0.219	
LSD	1.794	2.074	0.927	

Values followed by the same letter within columns are not significantly different according to the LSD listed at $\alpha \leq 0.05$.

* Disease severity ratings are based on a scale that a rating 0 = no lesions and 20 = total necrosis of the leaves due to the disease.

In the Jewel there was no significant ($P \leq 0.1994$) difference among any of the five treatments of copper sulphate application, probably due to the fact that there was very little to no bacterial infection recorded (Table 5). When the overall disease rating means are observed it becomes apparent that the lowest disease ratings were recorded for the plots receiving repeated applications of copper sulphate (treatment 3 and treatment 4) (Table 5).

The general linear models procedure indicated no significant ($P \leq 0.1217$) differences among varieties at week 16. In week 57 there was a significant ($P \leq 0.0028$) difference between the Honeoye and the other two cultivars. At week 62 and week 68 there was a significant ($P \leq 0.0013$ and $P \leq 0.0279$) difference between Honeoye and Jewel respectively, and a significant ($P \leq 0.0279$) difference between Cavendish and Jewel. Even though Honeoye did have higher disease severity ratings, there was no significant difference between the ratings of Honeoye and Cavendish throughout the study (Figure 2).

4.7 Overwintering Results for Field Trials

On 25 May 1996 the field trials were examined for the presence of *X. fragariae* symptoms on the new strawberry foliage emerging in both field trials. It appears that the bacterium overwintered on all the cultivars, except for the

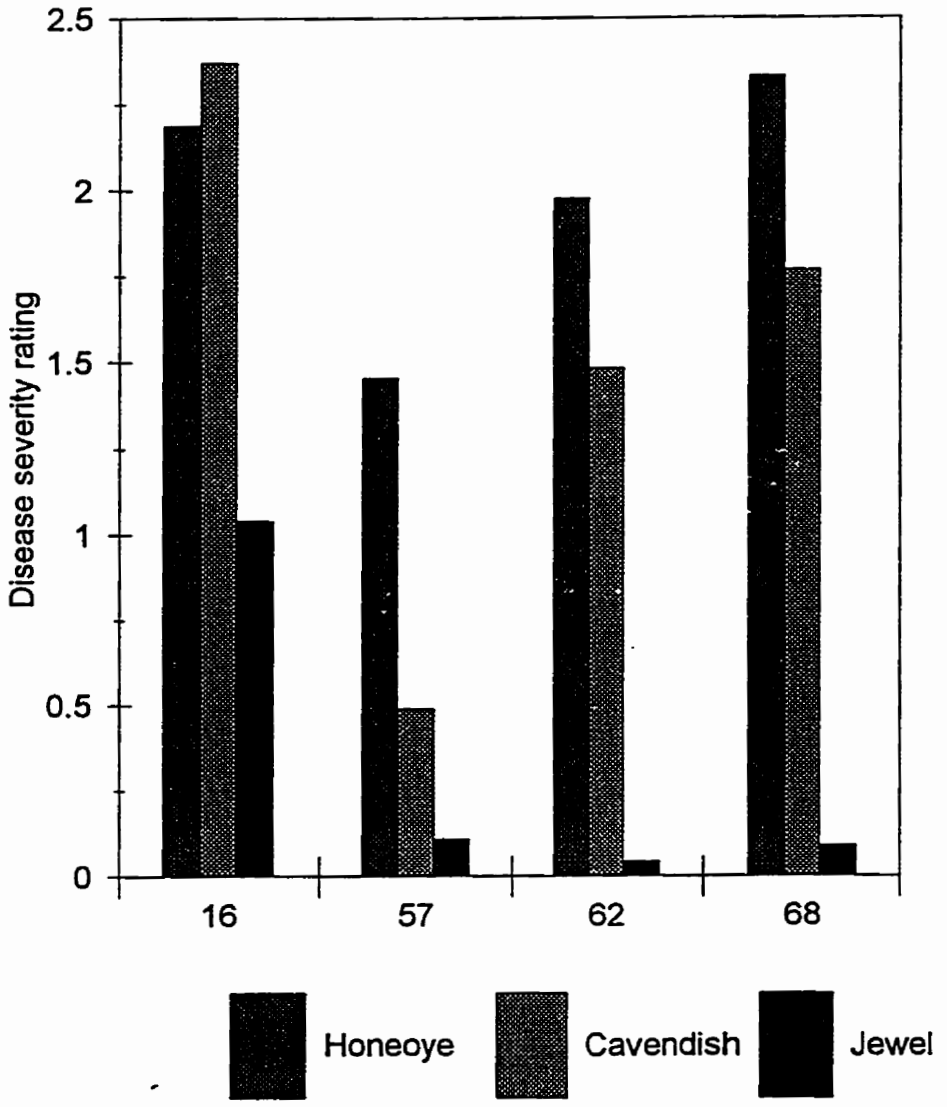

Figure 2: Mean disease ratings* for three strawberry cultivars in the copper sulphate trial over 168 weeks**.

*Disease severity ratings are based on a scale that 0 = no lesions present and 20 = total necrosis of the leaves due to the disease.

*LSDs of 0.4126, 1.1578, and 1.3605 for Honeoye, Cavendish, and Jewel respectively at a $\alpha \leq 0.05$.

Jewel cultivar which had very little infection present the previous growing season (Table 6). The presence of the bacterial infection was also visible in the copper sulphate trial indicating that the copper sulphate did not suppress the bacterium over the winter season (Table 7).

4.8 Growth Chamber Studies

Disease symptoms appeared on the infected plants which were inoculated with various dilutions of nutrient broth (N.B.):sterile distilled water. Symptoms were visible three days after inoculation. The dilutions of 20-70% N.B. had a few minute lesions present. After eight days all of the inoculated plants began to show angular lesions on the leaves. The 0% N.B. solution treatment had a few small (>1mm^2) spots on the leaves, whereas the 100% N.B. suspension had infected areas 5 mm^2 in size. After 11 days, distinct angular water-soaked lesions were present on plants receiving 0-60% N.B. suspension. The plants receiving 70-100% N.B. solution did not have distinct lesions, but had pale yellow areas along the leaf veins. After 14 days, it was obvious that visually the most distinct lesions were present on plants receiving 20-30% N.B. suspension. A range of symptoms were present on all plants receiving the various dilutions of N.B.:H_2O. There were no lesions or symptoms visible on the control plants used throughout the study.

Table 6: Incidence of angular leaf spot in the four blocks of the host cultivar trial in the year of establishment.

Cultivar	Incidence
Mic Mac	100%
Honeoye	100%
Cavendish	75%
Annapolis	75%
Jewel	0%
NY 113	100%

Table 7: Incidence of angular leaf spot in three strawberry cultivars after various applications of copper sulphate in the field.

Treatment	Disease Incidence % Cultivars		
	Honeoye	Cavendish	Jewel
1	50%	75%	0%
2	50%	75%	0%
3	50%	75%	25%
4	75%	75%	0%
5	75%	100%	0%

4.9 Temperature Study

In the temperature study, lesions appeared on the leaves approximately 72 hours after inoculation. The lesions appeared initially as dark green areas along the leaf veins. As time increased, lesions expanded to interveinal portions of the leaf and appeared translucent when held up to a light source. Lesions remained < $1cm^2$.

Once analysis began it was obvious that at 24 and 48 hour sampling times the numbers of bacterial colonies on the plates were all at or below a threshold count of 150 CFU/cm^2 leaf tissue. Since there was no difference between these two sampling times for any treatment, they were excluded from the statistical analysis. Statistical analysis was based on the remaining sampling times.

At 16°C, Honeoye and Jewel had similar levels of bacteria (Table 8). At 20°C, the bacterial count in Jewel was lower (359.0 CFU/cm^2 leaf tissue), whereas Honeoye had a high bacterial count (1122.0 CFU/cm^2 leaf tissue) (Table 8).

At 23.5°C the bacterial count for Jewel was low (189.0 CFU/cm^2 leaf tissue), and Honeoye's bacterial count was also lower than at 20°C (542.5 CFU/cm^2 leaf tissue). At 27°C the bacterial counts for both Jewel (150.0 CFU/cm^2 leaf tissue) and Honeoye (420.5 CFU/cm^2 leaf tissue) were lower than in the lower temperature settings (Table 8).

Statistically there was a significance ($P \leq 0.0052$) difference between the bacterial counts at 16°C and 20°C in

Table 8: The effect of temperature on the mean bacterial counts (CFU/ cm^2 leaf tissue) in Honeoye and Jewel cultivars.

Cultivar	Temperature (°C) 16	20	23.5	27	Mean for each cultivar
Honeoye (LSD=393.99)	735.50^{ab}	1122.00^{a}	542.50^{b}	416.00^{b}	704.00
Jewel (LSD=282.99)	671.00^{a}	359.00^{b}	189.00^{b}	150.00^{b}	342.25
Mean for each temperature	703.25	740.5	365.75	283.00	

Values followed by the same letter within rows are not significantly different according to the LSD listed at $\alpha \leq 0.05$.

Honeoye, and there was no significant difference among the bacterial counts at 16°C, 23.5°C, and 27°C. In Jewel, there was a significant ($P \leq 0.0025$) difference between bacterial counts at 16°C and the other temperature settings, and there was no significant difference occurring among the bacterial counts observed at 20°C, 23.5°C, and 27°C. Overall the Honeoye bacterial counts were higher than the bacterial counts observed for Jewel.

4.10 Moisture Study

The moisture study was repeated three times however no symptoms developed on the inoculated plants. The *X. fragariae* culture was re-inoculated back onto a strawberry plant and reisolated to ensure pathogenicity of the bacterium.

5. Discussion

5.1 Culture Isolation and Storage

The YDC medium seemed to be the best growth medium for *X. fragariae* cultures. Hazel and Civerolo (1980) studied the growth of *X. fragariae* on various types of media. They reported that YDC medium supported 3-5 times more viable cells at 24 hours and 5-7 times more cells at 48 hours than other media such as nutrient broth. The dextrose must be able to supply more carbohydates for the bacterium's growth as compared to the other media that do not contain this sugar. Hazel (1981) also stated that basal medium (supplemented with 0.5% casein hydrolysate, 0.1% yeast extract, and 1.0% dextrose) and YDC were both excellent plating media for *X. fragariae* since single colonies appear within 3-5 days of plating.

Even though YDC medium appeared to be the best plating medium for *X. fragariae*, this medium was not able to maintain the pathogenicity or vigour of this bacterium for long periods of time. SPA medium was able to maintain the pathogenicity of this bacterium for longer periods of time. Alippi *et al.* (1989) reported that *X. fragariae* colonies grew slowly on SPA. This slow growth may account for the lasting pathogenic properties of the bacterium.

5.2 Inoculation Method

It appeared that the most effective means of inoculation with *X. fragariae* was the spray inoculation method. In most of the literature inoculation of *Xanthomonas* spp. was performed using an atomizer sprayer or low pressure sprayer also.

The 3 mL of inoculum used to spray the strawberry plant seemed to be an adequate amount in order for infection to occur. The 3 mL of inoculum used was able to provide enough liquid to provide a medium of movement of the bacterium into the host tissues, and did not cause over water soaking of the tissues. Hazel and Civerolo (1980) and Hazel (1981) inoculated strawberry with *X. fragariae* using an airbrush. The inoculum was sprayed onto the lower leaf surface at 1 kg cm^{-2} and approximately 3 cm from the leaf for 2-4 seconds. Their results indicated that if the leaves were completely water soaked, indicated by a dark green color, there was localized, non-specific necrosis and poor development of lesions. They reported that spray infiltration was fast and easy, and many sites on a single leaflet could be inoculated.

The pin-prick method of bacterial inoculation was not successful in this study. It can however be used on any part of the host plant and has shown some success in other studies. There are no escapes in this method and in general placing plants in a humid chamber is not essential. The principle of this method is to injure the host at various

points with a group of fine needles and apply inoculum to the injured surface (Dhingra and Sinclair 1995). This method bypasses bacterial penetration through the stomata, rather it causes a wound in the epidermis and the bacteria are concentrated in the leaf mesophyll tissue (Graham *et al.* 1992). This may account for the necrotic lesions present near the point of injection on the inoculated plants. The bacteria may have been in such high concentrations that the surrounding tissue was damaged in the early stages of infection.

Kennedy and King (1962) inoculated strawberry leaf petioles with *X. fragariae* by means of needle puncturing, and reported that lesions first appeared around the wound, and then quickly spread up the petiole and along the large veins. The leaves then rapidly cupped downward and the petioles shrivelled and turned black, followed quickly by death. Thus this infection process was very rapid and did not represent natural infection processes for this disease.

The reason for such rapid and unpredictable infection by the pin prick method may be due to the fact that the injection of bacteria into leaf mesophyll bypasses the penetration through the epidermis. Thus the bacterium does not come in contact with the leaf-surface resistance mechanisms (Mazzucchi *et al.* 1973, Stall *et al.* 1982).

The disease symptoms on the inoculated plants were more prominent and natural when the plants were maintained under

plastic bags on the mist bench after inoculation. This procedure increased the relative humidity around the infected plant and seemed to enhance the process of infection. The misting of the plants before and after inoculation also seemed to enhance infection. In fact environmental conditions, such as moisture and relative humidity, are important factors that have to be considered when inoculating plants with such a bacterium.

When pathogens enter plants through stomata certain conditions such as moisture must be present in order for infection to occur. Water-congestion is an important characteristic in order for bacteria to infect and multiply within plant tissues. Under normal conditions the intercellular spaces of plants contain air, however in some instances these spaces fill with water and become congested. The bacteria can reach high populations in the intercellular spaces only when these are filled with water. The size of the host's intercellular spaces may also be influential in the infection process. If the intercellular spaces are not water congested then the pathogen will be drawn to the plant cell walls. Usually as infection progresses there is persistent water-soaking at sites of bacterial multiplication (Billing in Rhodes-Roberts and Skinner 1982). It is also believed that extracellular polysaccharides from some bacteria induce water-soaking in plant leaves of susceptible cultivars of certain hosts (El-Banoby and Rudolph 1979).

The duration of misting before and after inoculation therefore seems to be a very important factor for disease development. The longer the inoculated plant remains under water-congested conditions, more distinctive lesions will develop (Williams and Keen 1967, El-Banoby and Rudolph 1979, and Riker 1929).

It was also found that angular leaf spot development was dependent on the relative humidity in the growth chamber before and after inoculation. When the inoculated plants were maintained under the plastic bags on the mist bench more lesions developed. Williams and Keen (1967) reported that only minute lesions developed on plants incubated at 85 and 90% relative humidities and fewer lesions formed at 85 than at 90% relative humidity. Typical lesions developed however at 95 and 100% relative humidity. At 85 and 90% relative humidity chambers never accumulated free surface moisture, whereas those at 95 and 100% relative humidity accumulated droplets on leaf undersurfaces.

In this study symptoms tended to diminish when the plants were moved from the mist bench to the greenhouse probably due to the fact that the moisture and high humidity conditions were not provided in the greenhouse environment. However it should be noted that the mist bench temperature was 25.5°C and the greenhouse temperature was 26°C, which may have been too high for disease development to occur since *X. fragariae* prefers low temperatures near 20°C.

More lesions developed in this study when plants were placed in the dark before inoculation and then inoculated under light conditions. When the plants were maintained in the dark the stomata were closed and then when they were brought back out into the light the stomata would open and be ready for inoculation. Ramos and Volin (1987) reported that the number and size of leaf spot lesions were significantly reduced when the stomata were closed in plants grown and inoculated under dark conditions.

It was also reported that the stomatal frequency is higher in young expanding leaves, then decreasing and remaining constant in full or near fully expanded leaves (Ramos and Volin 1987). This may account for the fact that younger tissue is more susceptible to this bacterial infection than older foliage tissue. Stomatal frequencies also are usually lower in the adaxial surface of the leaf and higher in abaxial surface (Ramos and Volin 1987). Therefore this explains why more lesions developed when the bacterial suspension was sprayed on the undersurface of the leaves, rather than spraying on the upper leaf surface.

5.4 Host Cultivar Trial in the Field

It appeared that it took some time for the disease to progress and spread to the surrounding plants in the field. It was first thought that since the disease is very sensitive to environmental conditions, such as high temperatures and

moisture levels, that these factors may be inhibiting symptoms to develop or for the disease to spread throughout the plots. However, there was no evidence to support this since there was very little difference between the two growing seasons. Nevertheless the amount of precipitation in the two growing seasons was above normal (+22.6% deviation from normal) and may account for the fact that so many distinct water-soaked lesions developed on these selected cultivars in this study.

It can be concluded that the cultivar with the highest disease rating or highest susceptibility to *X. fragariae* in this study was Mic Mac. However after week 62 there was no significant difference among Mic Mac, Honeoye and NY113. It was not surprising to find that Honeoye or Mic Mac were susceptible. However it was anticipated that Honeoye would be the variety with the highest disease ratings. Several studies have reported that Honeoye is highly susceptible to this disease. No studies on the susceptibility of Mic Mac have been reported to date. Both Honeoye and Mic Mac had considerable mucus associated with diseased leaf surfaces. Maas *et al.* (1995) reported that Honeoye was highly susceptible to angular leaf spot.

The high disease levels in NY113 were unexpected. It was believed to contain some resistant properties. At first very few symptoms were visible, but as time progressed more distinctive lesions were present. Thus it appears that this

variety may not be an option for future work in a resistance breeding program in this environment. In contrast, however, Maas *et al.* (1995) reported that NY113 was relatively resistant to the angular leaf spot disease. Thus more research should be performed on the NY113 cultivar under various environmental conditions to determine its resistance levels.

The Cavendish and Annapolis cultivars had moderately low levels of disease throughout this study. Even though lesions were present there did not seem to be a threat of spread of this disease in these plots. However it is interesting to note that Annapolis has been reported to be a susceptible variety here in Nova Scotia (personal experience). Maas *et al.* (1995) reported that Cavendish was very susceptible to the disease.

Disease levels on Jewel were minimal throughout this study period. Even though the ratings were not significantly different from the ratings of Cavendish and Annapolis at week 68 (Table 3), Jewel did not produce the distinctive lesions that the other varieties did. Even in the overwintering results the bacterium was found in all cultivars except in Jewel in the spring of 1996 (Table 6-7). Maas *et al.* (1995) also reported that Jewel was relatively resistant to this disease. Therefore the Jewel cultivar may have potential in a breeding program for resistance to this bacterium, however more research should be performed for verification.

The cultivar Jewel tends to have a thicker, darker green leaf. This thicker leaf epidermis may account for some of its resistance. A thicker and waxier cuticle or epidermis may secrete more defensive biochemicals which aide in the destruction or blocking of an invading pathogen. The thicker epidermis may also be more lignified making it difficult for bacteria to invade the host tissues. This avenue of resistance should be looked into further and may present some opportunity for a breeding program for resistance with this Jewel variety and this particular strain of *X. fragariae*. The problem with a breeding program for this disease is that there may be several physiological races of this bacterium. The isolate used in this study was a very slow growing bacterium and was not very vigorous at times throughout the study. It is possible that there are strains of this bacterium which may be more aggressive and may overcome the plant resistance mechanisms that this variety may contain. Therefore future work must include the testing of different strains of *X. fragariae* on Jewel under several environments in order to verify the degree of resistance.

5.5 Copper Sulphate Trial

There was no significant difference recorded among cultivars at week 16, or for the 1995 growing season. The reason may be that it took time for the bacterium to get established and spread within the plots. However it is

interesting to note that the overall disease ratings for all three cultivars throughout the study were much lower than the ratings recorded in the variety trial for the same cultivars at the same time periods (not statistically analyzed). The copper sulphate may have been a factor in this observation. The copper sulphate may have been able to control the disease at its initial stages in the first growing season, which would account for the low numbers of infected plants being recorded. Therefore it can be concluded that the application of copper sulphate does seem to have a positive impact on the early establishment of angular leaf spot on strawberry. It was initially believed that the prebloom spray would be the optimum timing of spray due to the fact that it is at the bloom stage of the strawberry plant when the bacterium is likely to infect its host. It was thought that the copper sulphate would act as a protectant during this sensitive plant stage. However the results have indicated that this spray timing has very little impact on the disease and in fact had very little difference than the controls.

Copper sulphate, like most copper-based fungicides, is basically a protectant in action. Copper fungicides are efficient protectants because they are not deactivated by light and spread evenly over the crop surface, thus providing a stable coverage of residual fungicide which will protect the crop in case of repeated pathogen invasion (Mabbett

1984). However copper sulphate is insoluble and slowly available and therefore must be applied at the proper time and concentration to ensure protection.

It is known that the inhibitory effects of copper on bacteria are dependent on the concentration of free copper ions. Copper ions are mobilized from bactericide deposits by carbon dioxide in rain, by microbial exudates, and by solubilizing agents in the exudates from plant surfaces. The mobilized copper ions may be in a free form or chelated by organic compounds, in which case they lose toxicity to bacteria (Lee *et al.* 1993).

Since exudate was produced on the leaf surfaces by *X. fragariae* in this study, and there was rainfall and/or dew present or after copper sulphate application this may explain why complete control of the disease was not accomplished. The copper ions responsible for control on the strawberry leaf may have been washed off or mobilized by other compounds. This may explain also why lower disease severity ratings were recorded for repeated applications of copper sulphate. The strawberry plant is continuously producing new growth, in which the bacterium could infect. Since copper sulphate is a protectant, then repeated applications should be applied in order to protect new emerging plant tissue.

A problem with the use of copper sulphate as a bactericide is that there is a possibility of phytotoxicity to the crop due to the Cu^{2+} ions. Copper can build up in

soils through the accumulation of runoff after spraying and the decomposition of treated leaves (MaGuire 1988). The crop can absorb too much copper which can damage the size of the crop as well as the fruit production (Lee *et al.* 1993). In this study there were no phytotoxic effects observed on the plants even with repeated applications of copper sulphate. Allen (1989) reported that the uptake of copper is dependent on the pH of the soil. A pH of 6.5 or higher will increase the chances of phytotoxic effects developing (Payne *et al.* 1988). The soil in the field trials for this study had a pH of 5.4, therefore may have been acidic enough for phytotoxic effects not to develop.

In the overwintering results in the spring of 1996, the bacterium or signs of the disease were observed in all treatments except for the Jewel cultivar treatments. Thus the copper sulphate application the previous growing season failed to reduce or eliminate the disease in the following season. As the disease progresses, the bacterium infects inner membranes and tissues of the plant. Since copper sulphate is a protectant it may not be able to come in contact with the bacterium at this stage, thus would not have any affect on the disease development.

It terms of chemical control of this bacterial disease, repeated applications of the copper sulphate appears to provide adequate control. However there are some long term effect of copper sulphate application that should be

investigated before a recommendation is made. A continuous application of copper sulphate may lead to copper sulphate insensitive strains of bacteria to emerge. There is also the concern of copper ions building up in the soil to cause phytotoxicty to the crop.

5.6 Growth Chamber Studies

The 20-30% nutrient broth solution was used for the inoculation procedure instead of 100% water because the strain of *X. fragariae* used appeared very weak and slow growing. The nutrient broth provided enough nutrients to support the bacterium to enable to infect its strawberry host. The results indicated that symptoms were not affected by the use of the nutrient broth, therefore it was consistently used throughout the study. However if this study was to be repeated, the bacterium should be suspended only in sterile distilled water. Even though the nutrient broth did not seem to cause an effect on the inoculation procedure, contaminants may have been present in the inoculum suspension. If other organisms were present, they could out compete *X. fragariae* in the plant tissue and cause symptoms on the strawberry host not to develop.

5.7 Temperature Study

This study was performed numerous times due to problems arising not only in the data collection process but also in

the growth chambers themselves. Due to space constraints two growth chambers had to be used for this study. So the four temperature settings had to be performed at separate timings. There is some degree of error involved in the analysis initially due to the fact that different growth chambers were used and at different timings, but more error arose when after several replications were performed and it was discovered that a coolant leak had occurred in one of the growth chambers during the study. The method of analysis is also at question. When the infected tissue was ground into a slurry and diluted one must decide whether a sufficient sample was taken and analyzed.

The growth chambers used were not able to control the relative humidity inside the chambers so plastic bags had to be placed over each individual plant. Therefore it was difficult to mist the plants regularly to maintain adequate levels of moisture required for the infection process to occur. Due to time and plant material constraints the study could not be performed another time, however the existing results were examined to speculate on any patterns that may be present. Therefore discretion must be used when reviewing the results of this study.

At 16°C it appears that Honeoye and Jewel maintained similar levels of bacteria (Table 8). This similarity may be due to the fact that the temperature is too cool for bacterial colonies to increase and for a difference to be

detected.

At 20°C the bacterial count for the Jewel plants remained low, whereas a high bacterial count was observed for Honeoye (Table 8). This temperature seemed to support the growth of *X. fragariae* compared to the other temperature settings studied. This high bacteria count may reflect the fact that this temperature setting and cultivar produced the greatest numbers of translucent lesions visible on the leaves.

At 23°C and 27°C, bacterial counts declined in both cultivars. The higher temperature settings may have reduced the bacterial multiplication in the host's tissue. There were very few lesions recorded for these temperature settings also.

5.8 Moisture Study

This experiment was not completed due to the fact that symptoms did not develop on the plants after inoculation. It was initially believed that the bacterial culture lost its pathogenicity, however the bacterium was re-inoculated onto a strawberry host plant and reisolated to ensure its vigour and pathogenic properties.

After the plants were inoculated they were placed back into the mist chamber and subjected to different time periods of moisture. Once removed from the mist chamber a hot air dryer was used to dry off the existing moisture on the plant

surfaces to ensure the exact length of moisture available. It is believed that the hot air dryer was too hot and caused the water pathway into the host's tissues to be eliminated and also may have dried or killed the bacterium since it can not tolerate temperatures above 33 C. The force of the dryer may have also damaged the plant tissues.

In subsequent trials the hot air dryer was not used but still symptoms did not develop. The length of moisture durations may not have been long enough to cause infection to occur also. For infection of *X. fragariae* to occur, high humidity levels and certain levels of moisture must be present in order for water congestion of the strawberry tissues to occur.

Another explanation for lack of symptom development may be due to the fact that once the plants were removed from the mist chambers they were placed in growth chambers. These growth chambers were not able to maintain high humidity levels necessary for this disease to progress.

5. CONCLUSION

It can be concluded that the cultivar with the highest disease rating or highest susceptibility to *X. fragariae* in the cultivar trial was Mic Mac. However after week 62 there was no significant difference among Mic Mac, Honeoye and NY113. The cultivars Cavendish and Annapolis had moderately low levels of disease throughout the study. Jewel showed the greatest potential for resistance to this leaf spot disease. Even though the ratings for Jewel were not significantly different from the ratings for Cavendish and Annapolis at week 68, Jewel did not produce the distinctive lesions that the other cultivars produced. More research should be performed on this cultivar to pin-point its resistant properties.

In the copper sulphate trial it was concluded that higher disease ratings were recorded in the control plots, except in the cultivar Jewel which had very low disease ratings. This indicates that copper sulphate had some impact on the bacterium. However, there was no significant difference among the copper sulphate timing treatments. Nevertheless lower mean disease ratings were recorded in treatments 3 and 4, which were repeated applications of copper sulphate at the flower bloom stage. There were no phytotoxic effects observed on the trial plants.

The growth chamber studies proved that this bacterium is

difficult to work with under artificial conditions. In order for disease to develop, high relative humidities and a supply of available plant moisture are necessary to obtain successful infection.

Appendix

Table 1: Analysis of variance data for week 16 in the cultivar field trial.

Source	DF	Sum of squares	F Value	Pr>F
Model	8	26.2797	1.40	0.2748
Error	15	35.2912		
Corrected Error	23	61.5709		
Cultivar	5	16.3166	1.39	0.2843
Block	3	9.9631	1.41	0.2783

Table 2: Analysis of variance data for week 57 in the cultivar field trial.

Source	DF	Sum of squares	F Value	Pr>F
Model	8	99.3700	5.84	0.0017
Error	15	31.8829		
Corrected Error	23	131.2529		
Cultivar	5	98.3852	9.26	0.0004
Block	3	0.9848	0.15	0.9252

Table 3: Analysis of variance data for week 62 in the cultivar field trial.

Source	DF	Sum of squares	F Value	Pr>F
Model	8	226.4830	3.04	0.0302
Error	15	139.5942		
Corrected Error	23	366.0772		
Cultivar	5	183.7136	3.95	0.0174
Block	3	42.7694	1.53	0.2471

Table 4: Analysis of variance data for week 68 in the cultivar field trial.

Source	DF	Sum of squares	F Value	Pr>F
Model	8	337.1554	4.12	0.0088
Error	15	153.3293		
Corrected Error	23	490.4847		
Cultivar	5	310.7681	6.08	0.0088
Block	3	26.3872	0.86	0.4829

Table 5: Analysis of variance data for week 16 in the Copper sulphate field trial.

Source	DF	Sum of squares	F Value	Pr>F
Model	14	165.8922	2.52	0.0098
Error	44	206.5722		
Corrected Error	58	372.4644		
Cultivar	2	20.7532	2.21	0.1271
Timing	4	72.6745	3.87	0.0089
Cult.*Timing	8	69.7773	1.86	0.0916

Table 6: Analysis of variance data for week 57 in the copper sulphate field trial.

Source	DF	Sum of squares	F Value	Pr>F
Model	14	31.8238	1.66	0.0992
Error	44	60.0756		
Corrected Error	58	91.8994		
Cultivar	2	18.4196	6.75	0.0028
Timing	4	5.3724	0.98	0.4263
Cult.*Timing	8	10.8353	0.99	0.4555

Table 7: Analysis of variance data for week 62 in the copper sulphate field trial.

Source	DF	Sum of squares	F Value	Pr>F
Model	14	184.6746	5.20	0.0001
Error	44	111.5811		
Corrected Error	58	296.2557		
Cultivar	2	39.4567	7.46	0.0013
Timing	4	100.9763	9.95	0.0001
Cult.*Timing	8	46.4004	2.29	0.0384

Table 8: Analysis of variance data for week 68 in the copper sulphate field trial.

Source	DF	Sum of squares	F Value	Pr>F
Model	14	194.9811	2.03	0.0375
Error	44	301.7750		
Corrected Error	58	496.7561		
Cultivar	2	53.2993	3.89	0.0279
Timing	4	81.8961	2.99	0.0289
Cult.*Timing	8	60.4148	1.10	0.3809

Table 9: Analysis of variance data for Honeoye cultivar in the copper sulphate field trial.

Source	DF	Sum of squares	F Value	Pr>F
Model	4	147.9036	6.53	0.0001
Error	115	651.0329		
Corrected Error	119	798.9365		

Table 10: Analysis of variance data for Cavendish cultivar in the copper sulphate field trial.

Source	DF	Sum of squares	F Value	Pr>F
Model	4	47.5332	2.90	0.0250
Error	115	471.4678		
Corrected Error	119	519.0009		

Table 11: Analysis of variance data for Jewel cultivar in the copper sulphate field trial.

Source	DF	Sum of squares	F Value	Pr>F
Model	4	3.1773	1.53	0.1994
Error	115	59.8771		
Corrected Error	119	63.0544		

Table 12: Analysis of variance data for Jewel cultivar in the temperature study.

Source	DF	Sum of squares	F Value	Pr>F
Model	3	1688027.5000	5.78	0.0025
Error	36	3504520.0000		
Corrected Error	39	5192547.0000		

Table 13: Analysis of variance data for Honeoye cultivar in the temperature study.

Source	DF	Sum of squares	F Value	Pr>F
Model	3	2847425.0000	5.03	0.0052
Error	36	6793185.0000		
Corrected Error	39	9640610.0000		

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