KARI WHITLEY PARDA
Development and detection of bacterial soft rot of *Hosta* spp. Tratt. caused by *Erwinia carotovora* subsp. *carotovora*.
(Under the direction of JEAN L. WILLIAMS-WOODWARD)

A soft rot of hosta, caused by an unknown agent, was observed at a large wholesale nursery in South Carolina in 1999. Losses ranged between 80 and 90 percent in the cultivated varieties Aureo-marginata and Golden Tiara that had been chilled at 0°C in a refrigerated storage facility for 8-16 weeks to fulfill dormancy requirements. The causal agent of bacterial soft rot of hosta was determined to be *Erwinia carotovora* subsp. *carotovora.* Hosta plants spray-inoculated with suspensions of *E. carotovora* subsp. *carotovora* developed soft rot following 24 hours cold storage at 0°C. Spread of disease occurred during cold storage of dormant hosta rhizome divisions and through vegetative propagation. Bactericides and disinfectants tested were ineffective when used as a protectant prior to exposure to 0°C. Using polymerase chain reaction, specific primers ECC K and W were developed for the detection of *E. carotovora* subsp. *carotovora.*

INDEX WORDS: *Erwinia carotovora* subsp. *carotovora*, *Hosta* spp., Cold storage, Disease spread, Disease management

DEVELOPMENT AND DETECTION OF BACTERIAL SOFT ROT OF *HOSTA* SPP. TRATT. CAUSED BY *ERWINIA CAROTOVORA* SUBSP. *CAROTOVORA*

by

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DEDICATION

To my husband, Jon

with unlimited love

To my grandfather, Thomas Y. Whitley, Sr.

in memory and admiration

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CHAPTER 1

INTRODUCTION

Hosta spp. Tratt., commonly known as plantain lilies, are herbaceous perennial plants native to the temperate regions of China and Japan (1). Since the introduction of hosta into the United States in 1790 (4), they have become one of the most popular perennials across the country (9). Although the flowers are attractive, the principle appeal of hosta lies in the textural effect created by the foliage (10). In addition to the visual qualities that hosta contribute to the landscape, the genus is easily cultivated and has few insect or disease problems (5,10).

To keep up with consumer demand for this popular ornamental, nurseries in warmer regions of the United States, USDA plant hardiness zones 8B and below, are producing hostas to take advantage of the long growing season available. Unfortunately, the genus has a dormancy requirement that is achieved by a period of low temperature chilling before bud break and southern production areas cannot provide these requirements. As a result, hostas produced in southern nurseries are often slower to emerge in the spring and exhibit poor growth in comparison to plants grown in cooler climates (7). The use of a cold storage treatment, via coolers or refrigerated trailers, enables growers in climates warmer than the plant's southern limit of USDA Hardiness Zone 8 (2) to supply an artificial chilling period and complete the dormancy requirement of the plant.

The placement of perennial plants in cold storage to promote leaf and bud initiation is a relatively new practice in the nursery industry (8). This process allows the grower to mimic winter conditions, thus triggering normal physiological responses in the plant (3). Low-temperature storage of dormant perennials reduces dormancy requirements for earlier leaf emergence, completes vernalization for flower development, and increases the number of leaves per plant (6). Since hosta species are indigenous to cooler regions of China and Japan (2,5,10), they are well suited for cold storage. Nurseries have observed that a chilling period reduces time to bud break and produces a more uniform crop. Recently, in a study conducted by Keever, et al. (7), the positive influence of chilling, at 4° C for six weeks, was proven on hosta.

Many diseases can develop under conditions of cold storage. While there has been extensive postharvest research conducted on fruits and vegetables in cold storage, diseases of ornamental plants have been little studied.

A soft rot of hosta, caused by an unknown agent, was observed at a large wholesale nursery in Berkeley County, South Carolina in 1999. In preliminary investigations, it was noted that losses ranged between 80 and 90 percent in the culitvars ‘Aureo Marginata’ and ‘Golden Tiara’. This disease was causing maceration of the rhizomatous tissue following storage in a chilling facility. The epidermal tissue covering the fleshy roots remained intact, while the parenchyma tissue disintegrated into a watery rot. Aboveground symptoms were yellow, wilted leaves with water-soaked petioles that eventually collapsed at the soil line. Infected plants had a distinctive malodorous aroma once the rhizome began to rot.

The objectives of this research were to 1) identify the causal agent of soft rot on hosta, 2) develop an accurate method for detection of the pathogen, 3) determine the role of cold storage in the development of the disease, and 4) evaluate cultural and chemical strategies to prevent disease spread in nurseries.

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CHAPTER 2

LITERATURE REVIEW

CHARACTERISTICS OF HOSTA. *Hosta* spp. Tratt., commonly known as plantain lilies, are members of the Liliaceae family and indigenous to the temperate regions of China and Japan (1). Since their introduction to Europe and the United States in 1790, they have become one of the most popular perennials grown in the United States (52). Although their flowers are attractive, the principle appeal of hostas lies in the textural effect created by their foliage.

Hostas are divided into 40 species and consist of at least 3000 cultivars (21). Botanically, hostas are perennial plants consisting of a rhizomatous storage organ that produces wide, basal leaves and racemose flowers on a tall scape (22). Propagation is most common by rhizome division but can also be done by seed or tissue culture (1,54).

Plantain lilies grow best in moist, well-drained soil situated in light shade (22). They require low levels of nitrogen fertilizer, as excessive amounts minimize variegation in the leaves (3). Slugs and deer frequently destroy the foliage of hostas, while voles attack the fleshy roots. Disease is seldom a problem, but several fungi are capable of causing crown rot and leaf spot, including *Alternaria* sp., *Botrytis cinerea*, and *Sclerotium rolfsii* (5). Foliar nematodes (*Aphelenchoides fragaraie*) cause necrosis of leaf tissue and several viruses can also infect hosta (40,42).

SOFT ROT SYMPTOMS. Soft rot is a disease that degrades the fleshy tissue of fruits, vegetables and ornamentals (2). It frequently affects the underground storage organs of potato (*Solanum tuberosum* L.), iris (*Iris* spp.) and tulip (*Tulipa* spp.) as well as the fruits of apples (*Malus domestica* Mill.) and tomato (*Lycopersicon esculentum* Mill.) (13,24,29,30,45). This condition can occur in the field or in postharvest situations (32).

The primary symptom of soft rot is the maceration of fleshy tissue caused by enzymes released from the causal agent (14). The infected organ becomes soft and watery, and as a result, loses all structural integrity (9). Furthermore, a malodorous aroma may accompany this condition (18). Soft rot is caused by a bacterial or fungal agent.

BACTERIAL SOFT ROT. Soft rot bacteria enter host tissue through wounds, stomata and lenticels. (2,39). Actively growing bacteria reproduce intercellularly and release pectic enzymes (pectic lyases or polygalacturonases) (36,39) that enables the bacteria to dissolve the middle lamella of plant cells (13, 14). This leads to separation of the cells, which in turn causes the characteristic soft rot. Symptoms appear as water-soaked lesions on the surface of the infected organ that become discolored and increase in size as bacterial enzymes digest the tissue (11). Eventually, the cell wall disintegrates, the cell is plasmolyzed, and the tissue becomes macerated (20,39). Cell death occurs because the damaged cell wall cannot maintain the integrity of the cytoplasmic membrane (51). The epidermis, which is not affected by enzymatic activity, cracks and allows the liquefied mass to exit into the surrounding soil (18) which can spread the disease to other plants. The fleshy storage organ is transformed into a brown, watery substance (11) and often a foul odor is detected because of the release of volatile compounds.

Favorable conditions for soft rot development include high temperatures and relative humidity, poor-draining soils, and wounded plant material (8,39).

The primary causal agents of bacterial soft rot in living plant tissues are *Erwinia* spp. Bergey and *Pseudomonas* spp. Migula (2). These agents are now discussed in detail.

Erwinia. *Erwinia* spp., of the Enterobacteriaceae family, are the most destructive organisms involved in soft rot of rhizomes and tubers. This genus is responsible for soft rot of potato, iris, and many other important crops (6,18,24). *Erwinia* species and subspecies capable of causing soft rot are *Erwinia carotovora* subsp. *carotovora* (Jones 1901) Bergey, Harrison, Breed, Hammer & Huntoon 1923, *E. carotovora* subsp. *atroseptica* (van Hall 1902) Dye 1969, *E. carotovora* subsp. *betavasculorum* Thomson, Hildebrand & Schroth 1984, *E. carotovora* subsp. *wasabiae* Goto & Matsumoto 1987, *E. carotovora* subsp. *odorifera* Gallois, Samson, Ageron & Grimont 1992, and *E. chrysanthemi* Burkholder, McFadden & Dimock 1953 (53).

Erwinia spp. are rod-shaped bacteria with peritrichous flagella (7,37). The pectolytic *Erwinia* spp. are Gram-negative, oxidase negative, and facultatively anaerobic (53). *Erwinia* are mesophilic, preferring temperatures between 27 and 30°C, but can survive and grow in temperatures ranging from 5 to 35°C (2). To differentiate between species and subspecies of the genus, biochemical and physiological tests are conducted, including erythromycin sensitivity, indole production, growth at 37°C, and acid production from carbohydrate sources (53).

Fleshy storage organs are infected by direct contact with a diseased plant or by contaminated soil, water, or insects (13,18). Bacterial cells enter plant tissue through

wounds or lenticels and break down cellular structure. Above-ground symptoms usually appear first, and are characterized by yellow, wilted leaves and a wet, decayed stem at the base of the plant (25,45). The bacteria eventually destroy the rhizome or tuber, and continue to the next host.

Erwinia are capable of overwintering in infected tissue, insects, contaminated tools and occasionally in the soil (25). Members of this group of bacteria may survive in pond and river water and often survive as epiphytes, without causing disease, on host tissue (8,13). *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* are known to be seed-borne pathogens. *Erwinia carotovora* subsp. *carotovora* is transmitted on the seeds of pepper (*Capsicum annuum* L.), lupins (*Lupinus* spp.), tobacco (*Nicotiana tabacum* L.), and celery (*Apium graveolens* L.) (23,49). Larkspur (*Delphinium* spp.) and clover (*Trifolium* spp.) seeds can be infested by *Erwinia carotovora* subsp. *atroseptica.*

Controlling *Erwinia* spp. involves efficient cultural and sanitary practices. Infection of tubers and rhizomes can be deterred if wounding is prevented (2). This is unrealistic for plants that are propagated vegetatively, like hostas, since this type of reproduction involves stem cuttings or root division. When vegetative propagation is employed, knives, hands, and the work areas should regularly be sanitized to reduce spread of the pathogen. In addition, products that are going into storage should be dried and placed in storage areas with low relative humidity and adequate air ventilation. Under conditions of high humidity and stagnant air, even uninjured roots and fruits can become infected (45,61). Low oxygen concentrations also favor disease development because it reduces the plant's resistance to infection (61).

In the field, crop rotation with a non-host crop, such as grains or cereals, will eliminate some overwintering soilborne bacteria. Planting in well-drained soil also reduces the incidence of *Erwinia* soft rot (25,38).

Almost any plant with a fleshy organ is predisposed to *Erwinia* soft rot. Vegetable crops susceptible to *Erwinia carotovora* include carrot (*Daucus carota* var. *sativus* L.), sweet potato (*Ipomoea batatas* L.), garlic (*Alliuim sativum* L.), potato and onion (*Allium cepa* L.) (10,11,24,55). Furthermore, many ornamentals, such as iris, lily (*Lilium* spp. L.), ginger (*Zingiber officianle* Boehm.) and dahlia (*Dahlia* spp. Cav.) are affected by *Erwinia carotovora* (25).

Erwinia carotovora subsp. *atroseptica* is the causal agent of potato and delphinum blackleg (39). In potato, it is a systemic disease causing rotting of the tuber and base of the stem (56). The infection is perpetuated by the use of infected seed potatoes. Delphinium blackleg is soft rot of the stems and buds. Often, the stem will crack and exude bacteria. The spread of delphinium blackleg is caused by excessive irrigation and use of infected seeds (25,49).

Erwinia chrysanthemi is a common soft rot in tropical and subtropical climates. It affects fleshy leaves and stems more than underground storage organs, but can infect onion bulbs and potato tubers (25). Bacterial blight of chrysanthemum (*Chrysanthemum* spp. L.) is caused by *E. chrysanthemi*, which causes softening and collapse of the stems and roots (26). It is spread by vegetative propagation.

***Pseudomonas*.** Pseudomonads associated with soft rot are *P. marginalis* (Brown) Stapp. pathovars, *P. viridiflava* (Burkholder) Dowson, and *P. gladioli* Severini (25,53).

Pseudomonas spp. are rod-shaped bacteria with one to several polar flagella (36,53). Most pseudomonads produce fluorescent pigments on iron-deficient media (53). They grow in temperatures ranging from 4 to 48°C, but as mesophiles, they prefer temperatures between 27 and 30°C (2,11). Species are identified using the LOPAT classification system. This series of biochemical tests includes levan production, oxidase reaction, pectolytic activity, arginine dihydrolase, and hypersensitive reaction (HR) on tobacco leaves (53).

Pseudomonads infect fleshy tissue through wounds and are spread by contaminated soil, water and insects. Symptoms caused by *Erwinia* and *Pseudomonas* are similar, resulting from the same cellular degradation of fleshy organs by pectolytic enzymes (36). *Pseudomonas* does not have the host range of *Erwinia,* and tends to infect onion, potato, and beet (*Beta vulgaris* L.) most severely (7,24,55).

Control of *Pseudomonas* spp. depends on proper cultural practices. Injury to the bulb or tubers should be avoided to prevent infection (2). Water and humidity levels should also be monitored, as *Pseudomonas* spp. thrive under moist conditions (25). Crop rotation with a non-host species such as corn or wheat is recommended (11).

Pseudomonas marginalis is responsible for soft rot of onion and potato (12,43). It causes the characterisitic maceration of the tissue or bulb.

Pseudomonas gladioli pv. *allicola* causes a soft rot of onion. Symptoms of this disease include dark discoloration and softening of inner scales (39). *Pseudomonas gladioli* pv. *allicola* is a bacterium that attacks the corms of the Iridaceae family, such as gladiolus (*Gladiolus* spp. L.) and iris. This disease is characterized by the maceration of

fleshy tissue and yellow or red spotting on the surface of the corm. The corm eventually splits allowing the yellow bacterial mass to exude from the organ (18).

FUNGAL SOFT ROT. Fungal soft rot occurs when spores come in contact with wounds of fleshy bulbs, tubers and fruits. Spores germinate and the hyphae secrete the enzyme propectinase, which dissolves the middle lamella of plant tissues (2). These enzymes advance ahead of the mycelium, inducing cell separation and tissue structure deterioration. The hyphae then release cellulolytic enzymes that break down the cell wall to allow the hyphae to absorb nutrients from inside the cell. Fleshy tissue infected with soft-rotting fungi will initially appear water-soaked (25,57). However, as the propectinase and cellulolytic enzymes degrade the cells, the fruit or tuber will become brown, watery, and filled with mycelia. The fungus will continue to grow within the organ until the epidermis cracks (2).

The causal agents of fungal soft rot in subterranean organs of herbaceous plants are *Rhizopus stolonifer* (Ehrenb. *Ex* Fries) Vuill., *Pythium* Prigsh. spp. and *Sclerotinia sclerotiorum* (Lib.) de Bary. These agents are in detail below.

***Rhizopus*.** *Rhizopus* soft rot, caused by *Rhizopus stolonifer*, occurs on fleshy organs of fruits and vegetables in post-harvest conditions such as storage or transit (11). Sweet potato is the most economically important vegetable crop affected by this pathogen (9), but floral crops like tulip and gladiolus are also susceptible (2).

Rhizopus stolonifer is a zygomycete that exists in soil as a saprophyte. It has well-developed, aseptate hyphae that readily produce sporangiophores in sporangia. The sexual spore is the zygospore which serves as the overwintering structure of the fungus. When favorable conditions are present, the zygospore germinates and produces a

sporangium. When sporangiospores are released from sporangia, can eventually contact wounds on fleshy storage organs. The sporangia germinate and the hyphae secrete pectinolytic and cellulolytic enzymes, which liquefy the host tissue and cause soft rot. Once the epidermis cracks, the mycelium covers the tuber, rhizome or fruit with sporangiophores and sporangia. The presence of these structures gives the infected organ a white, downy appearance (2,25).

By properly handling rhizomes and bulbs to prevent wounding, the severity of *Rhizopus* soft rot can be reduced (25). Also, adjusting humidity levels in storage areas to a range of 72 to 84 percent will inhibit mycelial growth. *Rhizopus stolonifer*, unlike other fungi, does not grow well at high relative humidity (10,11). Sweet potato roots are temporarily held at warm temperatures and high relative humidity to promote the formation of a wound periderm. This protective tissue decreases water loss and increases host resistance to pathogens like *Rhizopus stolonifer* (32)

***Pythium*.** Carrot and potato are the crops most often associated with *Pythium* soft rot, but in Japan, soft rot of ginger caused by *Pythium zingiberum* Takahashi, often occurs in vegetative propagation (27). *Pythium* is a member of the Oomycota, the water molds, and is characterized by the formation of spherical, thick-walled survival spores known as oospores. The oospores germinate when conditions are favorable and produce white, rapidly growing hyphae. The mycelium produces sporangia, which germinate to produce a germ tube. The germ tube penetrates the fleshy host tissue and initiates infection. Alternatively, sporangia produce motile zoospores, which are chemotrophic and can locate host tissue. Pectolytic enzymes produced by the pathogen dissolve the middle lamella of host tissue and soft rot occurs. With the exception of suberin and lignin, which

the fungus cannot break down, the organ is macerated. Sporangia appear on the surface of the organ when the disease has progressed, giving the structure a downy appearance. (2, 24)

To control *Pythium* spp., good drainage and air circulation must be ensured. Treatment of bulbs with a copper fungicide prior to planting will prevent onset of soft rot. Crop rotation with non-host species is also recommended (25).

Sclerotinia. The fungus, *Sclerotinia sclerotiorum* (Lib.) de Bary, causes cottony soft rot and watery soft rot of carrot and tuber rot of potato (11,47). White mycelial growth grows appears on the outside of the infected storage organ and sclerotia develop from the mycelium The sclerotia become hard and black over time and serve as survival structures for the fungus. Tubers and roots that are affected by this disease have typical soft rot symptoms caused by pectolytic enzymes released by the mycelium (11).

Control of *Sclerotinia sclerotiorum* is difficult because the sclerotia remain viable in the soil for up to three years. Crop rotation and fungicide applications are recommended. Proper plant spacing and good soil drainage will also impede growth of *S. sclerotiorum* (25).

COLD STORAGE. The placement of plant products in cold storage has traditionally been reserved for harvested fruits and vegetables to extend their shelf lives (32). Low-temperature storage delays deterioration by slowing down metabolic processes that lead to senescence in harvested products (16). Apples, tomatoes and onions are examples of fruits and vegetables typically stored in low temperature conditions. Most horticultural food products are harvested at a juvenile stage when their moisture contents are high, therefore, cold storage is beneficial because it suppresses respiration and water loss (32).

Cold storage of herbaceous perennial plants is a relatively new practice in the horticultural industry (41). The purposes of low-temperature storage for ornamental plants are to reduce dormancy requirements for earlier leaf emergence, to complete vernalization or chilling requirement for flower development and to increase numbers of leaves and flowers per plant (28,48). Essentially, artificial chilling of perennial plants duplicates winter conditions, allowing growth of leaf and flower buds at an earlier date (17). While many perennial and biennial plants require vernalization to develop flower buds in the spring, the flower buds of hosta and other rhizomatous crops have already differentiated prior to dormancy. The chilling hours that are accumulated during this time are therefore, not referred to as the vernalization period, but as the chilling requirement. In plants that are vernalized, flower buds will not form if the chilling hours are not completely satisfied. In contrast, plants with preformed flower buds will almost always break bud in the spring, regardless of the chilling hours received. The consequences of inadequate chilling are sporadic flowering, reduced plant vigor, and unsychronized growth. (50)

Many plants from temperate regions benefit from cold storage. Armitage et al. (4) showed that rhizomes of *Oxalis adenophylla* Gillies and bulbs of *Ipheion uniflorum* Raf. were positively affected by chilling. The days to foliar emergence and flower were reduced significantly after exposure to low temperatures. Dutch iris (*Iris* x *hollandica*) and tulips exhibit the same response to chilling (16,59). Similarly, corms of *Freesia hybrida* Ecklon ex Klatt respond to low-temperature storage at 5°C for four weeks by flowering 20 days earlier than corms that were not preconditioned (62).

Additional benefits of cold storage include an increase in the number of leaves per plant and better crop uniformity. The average number of leaves in *Iris* x *hollandica* increase linearly as the number of days in low-temperature storage increases (17). The rate of emergence from dormancy in rhizomes of *Lysimachia clethroides* L. increases with cold duration and the degree of growth is more uniform throughout the crop (28).

Not all perennial plants respond well to low-temperature storage. Chilling injury expressed as water-soaking of tissue or abortion of flower buds (48), can occur in plants that descend from tropical and sub-tropical regions. Furthermore, plants that originate from tropical areas do not have a cold dormancy requirement and the application of low temperatures does not accelerate flowering or bud break (50). For example, tubers of *Zantedeschia elliottina* (W. Watson) Engl. and *Zantedeschia* 'Pink Satin' have no dormancy requirements and flower with or without cold storage (19). *Leucocoryne coquimbensis* F. Phil. bulbs perform better when stored at temperatures greater than 20°C, yielding more inflorescences and fewer aborted flower buds (34). Cold storage of strawberry plants (*Fragaria* x *ananassa* Duch.) at –1.5°C causes the crowns to produce less flowers per plant, which consequently results in fewer fruit (35).

The growth of a plant as affected by natural climatic changes in known as thermomorphogenesis (16). In species that originate from more temperate regions, an artificial cold period can be beneficial. By implementing cold storage, the chilling requirement of the bulb or crown is achieved, and the plant is able to begin flowering or some other physiological action (17). The ability of nurseries and greenhouses to use cold storage in the southeastern United States allows the cold dormancy requirements of

more temperate plants to be satisfied more expeditiously and completely than if left to the relatively mild winters of the region.

Since hosta species are native to cooler regions of China and Japan (3) this genus is well-suited for cold storage. Nurseries have observed that cold storage reduces time to bud break and produces a more uniform crop. In a recent publication, the merits of chilling in hosta were quantified (33). Plants stored at 4°C for four weeks emerged quicker and had greater plant vigor compared to hostas that received no chilling exposure.

OTHER FACTORS. While cold storage may provide the ideal environment for the causal agent of soft rot in terms of temperature, moisture levels and light, other factors could be influencing the spread of this disease. It is possible that altering cultural practices could control soft rot in hosta (2).

The use of contaminated potting media or containers may be providing the inoculum that is infecting the hosta rhizomes. By using steam-sterilized, high-porosity potting medium in new or disinfected containers, the source of the causal agent could be eliminated. In addition, using sanitized pruning shears may prevent the disease from spreading from one plant to another (5).

Proper handling of rhizomes could also hinder development of this disease. Planting at correct depths and discarding wounded roots could prevent pathogen invasion (54). Also, if the rot is fungal the soil could be treated with a fungicidal drench to prevent disease development.

VOLATILE COMPOUNDS. Soft rot indicative of *Erwinia* spp. often exhibit a distinctive malodor as a result of production of certain volatile compounds. Once deterioration of

plant tissue is apparent a strong scent can often be detected. Previous research has been conducted using potato to determine if monitoring for these compounds could lead to early detection of this organism. In these experiments gas chromatography was used to determine which volatile compounds were produced as a result of bacterial infection. Varns and Glynn (58) found that compounds present were a combination of volatiles being produced by the causal organism, the host, and the host-pathogen interaction. While the infected potatoes produced elevated levels of many compounds compared to the control, the most notable increases were observed for 2-butanone. In another study, the primary compound expressed was 3-hydroxy-2-butanone (60). Potatoes infected with *Erwinia* spp. also expressed raised levels of ethanol, methanol and acetone (58,60). In a more recent study, infected potato tubers were analyzed and results indicated elevated levels of ammonia, ethanol, and 1-butanol (31). It was stated in the same report that monitoring ammonia levels is inefficient using a cryogenic trap and it is speculated that levels of ammonia produced by *Erwinia*-infected potatoes may be even higher then recorded. The presence of ammonia would account for the unpleasant odor associated with this disease.

Another study examining the volatile profiles from potatoes inoculated with *Erwinia carotovora* subsp. *carotovora* also showed that 2-butanone appeared in the analysis of inoculated potatoes, but not in the control (44). This experiment found greater differences in the amounts of dimethyl disulfide and pentane than in 2-butanone and classified the latter as a “background compound” since low levels were detected. Although most of the research on detection of *Erwinia* spp. on storage organs has been done on potato, it is possible that this technique could be used as a method for early, non-

invasive detection of hosta. Using a gas chromatograph to analyze the composition of the compounds formed during host-pathogen interactions, the presence of *Erwinia* spp. could be detected early in disease development. One candidate compound that can be monitored is 2-butanone. Although it is present in minute quantities in infected plants, it could provide an early indication of infection. The presence of dimethyl disulfide becomes apparent only after advanced decay occurs on the rhizome and is the result of secondary pathogens (44). Crop damage would have to be severe before this compound could be used as an indicator.

While the use of this technique would be beneficial in nurseries where problems with *Erwinia* spp. are more prone to occur, it is unlikely that the production of volatile compounds would be monitored in these situations. It would be difficult to collect volatile profiles from plants that are not in an enclosed area and the expense of periodic sampling would be unjustified. This technology would be effective using plants in cold storage or tissue culture labs. In cold storage, volatile profiles could be obtained easily because of the large number of plants concentrated in an enclosed space. This situation mimics potato storage warehouses where previous research has been conducted. Tissue culture laboratories, where many hosta cultivars are produced, could profit more by using gas chromatography because *Erwinia* spp. can be spread via this method of propagation. Therefore, it would be advantageous for laboratories to be able to detect the presence of *Erwinia* spp. before deterioration occurs.

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CHAPTER 3

EFFECT OF COLD STORAGE TEMPERATURE ON THE DEVELOPMENT OF *ERWINIA CAROTOVORA* SUBSP. *CAROTOVORA* IN *HOSTA* SPP.

Hosta spp. Tratt., commonly known as plantain lilies, are herbaceous perennial plants native to the temperate regions of China and Japan (1). Since the introduction of hosta into the United States in 1790 (10), they have become one of the most popular perennials across the country (20). Although the flowers are attractive, the principle appeal of hosta lies in the textural effect created by the foliage. In addition to the visual qualities that hostas contribute to landscapes, this genus is easily cultivated and has few insect or disease problems (11, 23).

Nurseries in the southeastern United States produced $9 million hostas in 2000 (17). Hosta has a dormancy requirement that must be fulfilled by a period of low temperature chilling before bud break can occur and southern production areas are often unable to satisfy this requirement (14). As a result, hostas produced in southern nurseries have slower spring emergence and exhibit poor growth in comparison to plants grown in cooler climates (14). Cold storage in coolers or refrigerated trailers may enable growers in warmer regions to satisfy dormancy requirements of the plant.

Cold storage of perennial plants to promote leaf and bud initiation is a relatively new practice in the nursery industry (16). This process allows the grower to mimic winter conditions and trigger normal physiological responses in the plant (8). Low-

temperature storage of dormant perennials reduces dormancy-breaking requirements for earlier leaf emergence, completes vernalization or chilling requirement for flower development, and increases the number of leaves per plant (6, 12, 19). Since hosta species are indigenous to cooler regions of China and Japan (2) they are well suited for cold storage. Keever et al. (14) observed that a chilling period of 4°C for four weeks reduces time to bud break and produces a more uniform crop.

Many postharvest and soft rot diseases develop under conditions of cold storage (13). While there has been extensive postharvest research conducted on fruits and vegetables in cold storage, diseases of ornamental plants have not been studied (13). A soft rot caused by an unknown agent was observed at a large wholesale nursery in Berkeley County, South Carolina in 1999 on hosta following storage in a chilling facility. Losses within the cultivars Aureo-marginata and Golden Tiara ranged between 80 and 90 percent. The epidermal tissue covering the fleshy roots remained intact while the parenchyma tissue disintegrated into a watery rot. Aboveground symptoms were yellow, wilted leaves with water-soaked petioles that eventually collapsed at the soil line. Infected plants had a distinctive malodorous aroma once the rhizome began to rot. Bacterial soft rot caused by *Erwinia* spp. has been reported but species identification was unknown (9).

The objectives of this study were to 1) determine the causal agent of soft rot on hosta, 2) determine the role of cold storage in disease development and 3) identify temperatures that satisfy chilling requirements without causing disease.

MATERIALS AND METHODS

Identification of Causal Agent. In November 1999, six cultivars of infected hosta (Honeybells, Golden Tiara, Royal Standard, Aureo-marginata, Antioch, and Shade Fanfare) were collected from a wholesale nursery in South Carolina. Forty rhizomes of each cultivar were potted into 3.8 liter plastic containers in a greenhouse and irrigated daily. Observations of symptom development were recorded and isolations onto nutrient yeast dextrose agar (NYDA; 23 g nutrient agar, 5 g yeast, 10 g dextrose, 1000 ml distilled water) were made from infected plants by washing affected plants in sterile water to remove soil debris, comminuting excised tissue in sterile distilled water (SDW), and incubating streaked plates at 30°C for 48 hours.

Bacterial isolates were identified by pectolytic activity on modified crystal violet pectate medium (24), fluorescence on King's medium B, anaerobic growth (22), and Gram staining (22). Isolates were maintained at -80°C in a nutrient broth-glycerol mixture (nutrient broth containing 20% glycerol, mixed after autoclaving broth).

Tissue-cultured plants of *Hosta* x 'Suzanne' (Agri-Starts, Inc., Apopka, FL) were stab-inoculated in the leaf whorl with 10 µl of a 10^8 colony forming units per milliliter (CFU/ml) bacterial suspension of selected isolates (standardized using a spectrophotometer [Spectronic 20+, ThermoSpectronic, Rochester, NY] to achieve 0.3 optical density). Plants were incubated at 100% humidity at 27°C for seven days or until symptoms developed. An *Erwinia* species was re-isolated in pure culture from the symptomatic tissue confirming it as the causal agent. *Erwinia* species identification was conducted using Biolog Microstation System and Biolog GN Database (Biolog, Inc., Haywood, CA) to evaluate substrate utilization of the pathogenic bacterium.

Additionally, fatty acid methyl esters (FAME) analysis (21) was conducted by Dr. Ron Gitaitis (University of Georgia, Tifton, GA) to further characterize the bacterial isolate to the species level. Finally, polymerase chain reaction (PCR) was conducted using universal primers and a primer set specific to *Erwinia carotovora* subsp. *atroseptica* (6).

Inoculum source. Irrigation water and hosta foliage were collected from the nursery in October 2000. Irrigation water (collected from sprinkler heads in hosta production blocks) and surface runoff (collected after irrigation) were used for 10-fold serial dilutions which were plated onto NYDA amended with cycloheximide (100 μg/ml; Sigma Chemical Co., St. Louis, MO) and incubated for 48 hr at 30°C. Putative colonies were selected and purified by sub-culturing. Bacterial isolates were identified using selective media, Gram-test, oxidase test and Biolog (22).

Foliar tissue was randomly removed from 20 hosta blocks (including 16 cultivars) and placed separately into sterile plastic bags. Each sample represented a single cultivar from one block of plants and contained 25 leaves from each block. All samples were processed within 12 hours of collection. Leaves from each sample were cut into 4 cm^2 pieces and 5 g sub-samples were selected for leaf washing. Leaf sub-samples were suspended with 200 ml of 0.1% peptone phosphate buffer (J.T. Baker, Phillipsburg, NJ)in a sterile 500-ml flask and agitated on a wrist-action shaker (Burrell Corp.; Pittsburgh, PA) for 30 min. Ten-fold serial dilutions were spread-plated onto cycloheximide-amended NYDA plates and incubated at 30°C for 48 hr. Plates were observed for growth, and selected colonies were identified using crystal violet pectate medium (24) and Biolog.

Influence of temperature on development of soft rot. Bacterial suspensions of 10^2, 10^4 and 10^8 CFU/ml were prepared by flooding 24-hr-old *Erwinia carotovora* subsp. *carotovora* cultures grown on NYDA at 30°C with SDW. Using a chromatographic sprayer, bacterial suspensions were applied, separately, to tissue culture-produced plants of *Hosta* x 'Suzanne' (Agri-Starts, Inc., Apopka, FL) until runoff occurred (~2 ml/plant). SDW-sprayed plants served as a control. The foliage dried for 24 hr to allow for possible epiphytic colony establishment.

Plants sprayed with SDW and each bacterial concentration were separated into three temperature treatments (0, 2, or 4°C). For each temperature treatment, 18 plants per replication were placed in sterile plastic bags and held for 24, 48 or 72 hr at 0, 2, or 4°C in separate Percival coolers (Percival Scientific, Inc, Perry, IA). Upon removal from cold storage, plants were potted into 0.35L plastic containers filled with Premier Pro-Mix (80% peat moss, 12% perlite, 8% vermiculite; Premier Horticulture, Red Hill, PA) and placed in the greenhouse in a randomized complete block design. Plants were irrigated daily and day and night temperatures were maintained at 27°C and 18°C, respectively. Plants were observed for two weeks for soft rot development and water-soaked tissue was tested for the presence of *E. carotovora* subsp. *carotovora.*

The experiment was repeated and data were analyzed by GLM-procedure following square root data transformation using SAS statistical software (SAS version 6.12, SAS Institute, Inc., Cary, NC). Comparison of means was by Tukey's HSD means separation test.

RESULTS

Identification of causal organism. The pathogenic bacterium was identified as an *Erwinia carotovora* subsp. *carotovora* using fatty acid methyl esters analysis (FAME) and Biolog GN Database with similarity indexes of 0.839 and 0.797, respectively. Type-isolates of *Erwinia chrysanthemi* and *E. carotovora* subsp. *atroseptica* (obtained from Dr. David Norman, University of Florida, Apopka) also proved to be pathogenic on hosta.

Inoculum source. The pathogen was recovered in low concentrations (10^0 -10^2 CFU/ml) from irrigation water, surface water, and on asymptomatic hosta foliage. *E. carotovora* subsp. *carotovora* was detected from all irrigation samples and 9 of 20 foliage samples. (Table 3.1)

Influence of temperature on development of soft rot. There was no statistical difference between the repeated tests (P=0.8070) therefore data are presented as pooled means. No disease occurred, regardless of bacterial concentration applied, unless the plants were held at 0°C for at least 24 hours (Figure 3.1). Chilling temperature significantly affected disease development (P=0.0001). Plants stored at 2 and 4° C showed no decline or soft rot symptoms, regardless of inoculation concentration. Typical soft rot disease symptoms were maceration of the leaf tissue, presence of bacterial ooze and degradation of root tissue. Non-inoculated control plants incubated at 0° C exhibited some leaf injury indicative of cold damage including a burn or necrosis of tissue from the leaf margins towards the petiole. In addition, some leaves initially showed water-soaking, but no disease development occurred.

While not statistically significant, disease incidence increased quadradically with an increase in bacterial concentration. Disease severity was consistent, regardless of

Table 3.1. Average population density of *Erwinia carotovora* subsp. *carotovora* detected in irrigation water, surface runoff, and healthy hosta leaves from a container-production nursery in South Carolina

Water Source	Block designation[y]	Average CFU/ml [z]
Irrigation	400	1.9×10^1
Irrigation	7702	1.1×10^1
Runoff	400	2.9×10^2
Cultivar		
Frances Williams	7702	4.5×10^1
Patriot	7702	3.1×10^1
Whirlwind	7702	2.0×10^1
Aureo-marginata	7702	1.7×10^1
Antioch	400	9.8×10^0
Aureo-marginata	400	7.2×10^0
Minuteman	7702	6.5×10^0
Francee	7702	4.8×10^0
Sea Thunder	7702	3.7×10^0
Sum and Substance	400	-
Sum and Substance	7702	-
Francee	400	-
Great Expectations	400	-
Hosta elata	400	-
Summer Music	7702	-
Hoosier Harmony	7702	-
Striptease	7702	-
Great Expectations	7702	-
Queen Josephine	7702	-
Patriot	7702	-

[y] Production area from which sample was obtained.
[z] Average detected colony forming units per milliliter (CFU/ml) of *Erwinia carotovora* subsp. *carotovora*

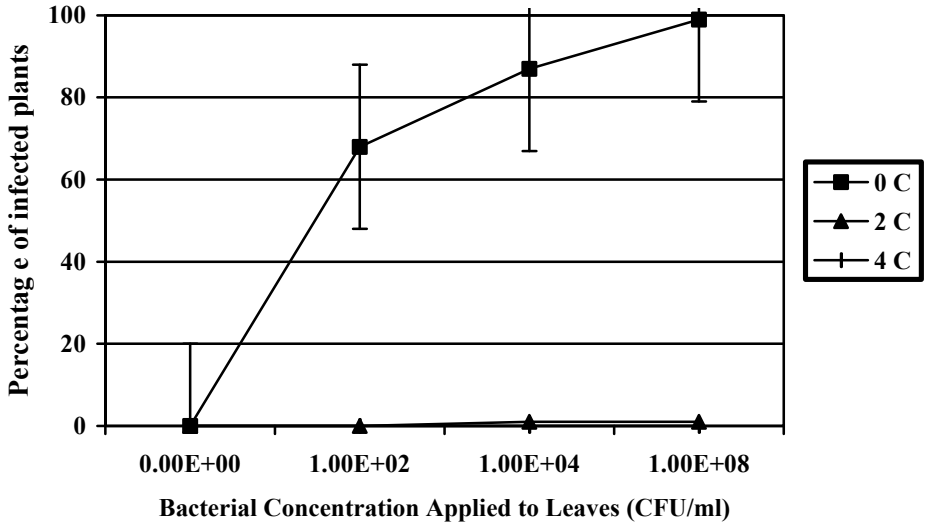

Figure 3.1. Percentage of *Hosta* x 'Suzanne' plants spray-inoculated with three concentrations of *Erwinia carotovora* subsp. *carotovora* or sterile distilled water that developed bacterial soft rot following incubation at 0, 2, or 4º C for 24 hr.

inoculum applied. Sixty-eight percent of plants sprayed with 10^2 CFU/ml of inoculum developed bacterial soft rot while 87% of plants inoculated with 10^4 CFU/ml and 99% of plants inoculated with 10^8 CFU/ml became infected.

DISCUSSION

Erwinia carotovora subsp. *carotovora* is endemic to many water sources (18) and has been recovered from irrigation ponds, rivers, oceans, aerosols, and surface-water (3). Cappaert et al. (3) recorded mean surface-water levels of *E. carotovora* subsp. *carotovora* up to 74.8 CFU/ml on asymptomatic plants irrigated with well water. *E. carotovora* subsp. *carotovora* was detected within irrigation water from the nursery's recycled irrigation water retention
pond, and this is most likely the source of *E. carotovora* subsp. *carotovora* contamination of hosta foliage.

E. carotovora subsp. *carotovora* is also often an epiphyte, residing on foliage and establishing populations, not causing disease until the plant is compromised by another disease or environmental factor (18). The bacteria concentrate along the midrib and petiole of a leaf and tend to survive better on senescent leaves (18). A small population will survive on the leaf surface and populations will rapidly increase under favorable conditions (15). Within the sampled nursery in this study, almost 50 percent of the asymptomatic hosta foliage was contaminated with detectable *E. carotovora* subsp. *carotovora* populations.

When soft-rotting *Erwinia* species are present on injured plant tissue, they use a method of bacterial communication known as "quorum-sensing" to perceive population size and regulate pathogenesis (7). Individual cells of bacteria release a diffusible

autoinducer compound, *N*- acyl-homoserine lactone, which allows the bacteria to distinguish population density. *N*-acyl-homoserine lactone leaks through membranes and accumulates in the environment, such as intercellular leaf space. When plant cell membrane integrity is compromised due to wounding and solutes leak from these cells, soft rot *Erwinia* species recognize that the plant is injured and the colony will increase in size. There is a minimum population density that must be achieved before the genes for maceration are expressed within the individual bacterial cells causing disease. As a result, the bacteria works like a multi-celled organism, producing extracellular pectinases and initiating disease (4, 7).

It is possible that injury to the plant caused by cold damage in storage is allowing epiphytic populations of bacteria to enter the plant and cause soft rot of the tissue. Through observations in the field and in records from the Plant Disease Clinic at the University of Georgia (Athens, Georgia), it has been noted that soft rot of ornamental rhizomatous plants such as hosta, iris (*Iris* spp. L.), and daylily (*Hemerocallis* sp. L.) often occurs following a late freeze in the spring or early freeze in the fall. These plants are not hardened-off and the succulent foliar tissue is, therefore, susceptible to freeze injury. These water-soaked leaves leak solutes that attract epiphytic populations of *Erwinia carotovora* and the damaged tissue provides an entryway for the bacteria to invade (7, 15, 18).

The temperatures of 0, 2 and 4°C are temperatures used by nurseries and tissue culture laboratories for storage, as well as satisfying the dormancy requirement of dormant perennial plants. None of the hosta plants held at 2 and 4°C developed bacterial soft rot regardless of storage duration, therefore storage temperature has a role in disease

development. Bacterial soft rot killed 68 to 99 percent of hosta plants when held at 0°C for a minimum of 24 hr. The epiphytic populations established on the foliage of inoculated plants utilized the freeze-damaged tissues as an infection court. Also, the solutes released by the damaged plant cells, especially once the plants were exposed to warmer temperatures in the greenhouse, encouraged bacterial populations to increase and caused disease symptoms to develop. (7, 18)

From this study, it has been determined that bacterial soft rot of hosta caused by *E. carotovora* subsp. *carotovora* can be prevented by storing and maintaining plants at temperatures above freezing. Even when epiphytic bacterial populations are relatively high, soft rot does not occur unless prior injury to the plant has transpired. It has been demonstrated the chilling hosta at 4° C satisfies dormancy requirements (14) and bacterial soft rot is not likely to develop if this temperature is maintained throughout the entire chilling period.

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CHAPTER 4

SPREAD AND MANAGEMENT OF BACTERIAL SOFT ROT OF *HOSTA* SPP. CAUSED BY *ERWINIA CAROTOVORA* SUBSP. *CAROTOVORA*

Hostas (*Hosta* spp. Tratt.), members of Liliaceae, are herbaceous perennial plants native to China and Japan (1). Over 3000 cultivars are available in the nursery trade providing consumers with a diversity of foliage colors, textures, larger flowers and fragrance (7). Hostas have few disease or insect problems (9) and are easily cultivated in shady sites. As a result, hostas are one of the most popular perennials in the United States (15).

Although nurseries in the southeastern United States produced $9 million hosta in 2000 (12), hostas grown in southern nurseries often have reduced vigor and exhibit a lack of uniformity compared to plants produced in cooler climates because the low-temperature chilling requirement needed to complete dormancy is not satisfied (10). Cold storage via refrigerated coolers can provide an artificial chilling period that fulfills the dormancy requirement of hosta (5). Keever et al. (10) demonstrated that a 4-wk chilling period at 4° C reduces time to bud break and standardizes crop development.

In 1999, soft rot on hosta caused by *Erwinia carotovora* subsp. *carotovora* (Jones 1901) Bergey, Harrison, Breed, Hammer & Huntoon 1923, was observed at a large wholesale nursery in Berkeley County, South Carolina following a period of cold storage. The fleshy parenchyma tissue of affected hostas was macerated while the epidermal layer

of the rhizomes remained intact. If leaves emerged, water-soaked lesions developed on petioles causing the leaves to collapse at the soil line. A malodorous aroma was evident as disease progressed. Losses of 80 to 90 percent in cultivars Aureo-marginata and Golden Tiara were observed following storage in the chilling facility. While bacterial soft rot of hosta has been reported (6), losses of this extent had previously not been documented.

Chilling healthy hosta plants with epiphytic populations of *E. carotovora* subsp. *carotovora* at or below 0°C results in soft rot development (13). Depending on bacterial concentration applied, 68 to 99 percent of plants chilled at 0°C developed bacterial soft rot.

The objectives of this study were to 1) determine if spread of *E. carotovora* subsp. *carotovora* occurs in cold storage and vegetative propagation and 2) identify chemical treatments to manage epiphytic populations of *E. carotovora* subsp. *carotovora* on hosta plants prior to cold storage.

MATERIALS AND METHODS

Spread in cold storage. Dormant *Hosta fortunei* 'Aureo-marginata' plants with well-developed rhizomes were obtained from a wholesale nursery in Athens, Georgia. Dormancy was maintained by storing plants at 4°C. Soil was washed from ten rhizomes to be used as a primary inoculum source. Rhizomes were surface disinfested with 0.525% NaOCl for 2 min and rinsed with sterile distilled water (SDW). A wound was created by dividing each rhizome with a sterile knife. Rhizomes were then submerged in a 10^8 colony forming units per milliliter (CFU/ml) *E. carotovora* subsp. *carotovora* for 30 seconds and dried overnight. The suspension was prepared by flooding cultures of *E.*

carotovora subsp. *carotovora* isolate 83, grown at 30°C for 24 hr on nutrient yeast dextrose agar (NYDA; 23 g nutrient agar, 5 g yeast, 10 g dextrose, 1000 ml distilled water) with SDW and standardizing the suspension using a spectrophotometer (Spectronic 20+, ThermoSpectronic, Rochester, NY) to achieve 0.3 optical density at 600 nm.

Infected rhizomes were placed at 0°C for 24 hr in a Percival cooler (Percival Scientific, Inc., Perry, IA). Upon removal, rhizomes were potted into 3.8 L plastic containers filled with Premier Pro-Mix (80% peat moss, 12% perlite, 8% vermiculite; Premier Horticulture, Red Hill, PA) and placed in the greenhouse until disease developed. Bacterial isolates were isolated from samples of infected tissue and Biolog Microstation System and Biolog GN Database (Biolog, Inc., Haywood, CA) were used to positively identify the causal agent as *E. carotovora* subsp. *carotovora*.

Soil was washed from the rhizomes of additional plants and disinfested as previously described. Plants were inspected for evidence of natural soft rot infection and questionable plants were discarded. All rhizomes were divided using a sterile knife to create an entryway for bacterial infection.

Five healthy rhizome divisions were placed in a sterile plastic bag with either one *E. carotovora* subsp. *carotovora*-infected rhizome or an uninfected rhizome. Moist peat moss (Premier Horticulture, Red Hill, PA) was added and each bag was sealed. Each treatment had five replications. All plants were incubated for 2 wk at 0° C in a Percival cooler.

After cold storage, all rhizomes were potted into 0.35 L plastic containers filled with Premier Pro-Mix and placed in the greenhouse in a completely randomized design.

Plants were irrigated daily and day and night temperatures were held at 27° C and 18° C, respectively. Plants were observed over 2-wk for development of soft rot and rated using a 1 to 6 disease rating system where 1= no disease; 2= some localized decay at point of division, plant overcame; 3=some root decay, but plant is growing; 4= most roots decayed, limited foliage growth; 5= water-soaked leaves, foliage collapses at crown, root decay; 6=complete death, plant does not emerge.

E. carotovora subsp. *carotovora* was isolated from select plants and confirmed using Biolog Microstation System, Biolog GN Database, and crystal violet pectate medium (17. The experiment was repeated and data were analyzed by GLM-procedure using SAS statistical software (SAS version 6.12, SAS Institute, Inc., Cary, NC).

Spread of *E. carotovora* subsp. *carotovora* by vegetative propagation. *Hosta fortunei* 'Aureo-marginata' plants were infected by submerging clean, surface-disinfested rhizomes into a 10^8 CFU/ml *Ecc* bacterial suspension as previously described. Soil was washed from additional hosta rhizomes and rhizomes disinfected with 0.525% NaOCl for 2 min. The experiment consisted of two treatments with five replications per treatment. An *E. carotovora* subsp. *carotovora*-infected plant or an uninfected plant was used to "inoculate" the propagation knife by cutting through the plant tissue. The knife was then used to sequentially divide five rhizomes in a series. Between replications, the knife was sterilized to prevent contamination.

After division, plants were potted into 0.35 L sterile plastic containers filled with Premier Pro-Mix and placed in the greenhouse. Plants were irrigated daily and day and night temperatures were held at 27°C and 18°C, respectively. For two weeks following division, plants were observed for bacterial soft rot development. *E. carotovora* subsp.

carotovora infection was confirmed through isolations and analysis using Biolog Microstation System and Biolog GN Database. The experiment was repeated and data were analyzed by GLM-procedure using SAS statistical software (SAS version 6.12, SAS Institute, Inc., Cary, NC).

***In vitro* bactericide/disinfectant sensitivity assay.** *E. carotovora* subsp. *carotovora* isolate 83 was grown for 24 hr at 30° C on nutrient yeast dextrose agar (NYDA; 23 g nutrient agar, 5 g yeast, 10 g dextrose, 1000 ml distilled water). Plates were flooded with SDW and diluted to a concentration of 10^8 CFU/ml. Twenty-five plates of NYDA were spread-plated with 100µl bacterial suspension and allowed to dry at room temperature for 2 hr.

Using a sterile cork borer, six 7-mm diameter plugs were removed from each plate at equidistant points. Wells were filled with 100 µl of selected bactericides and disinfectants and plates were incubated at room temperature for 24 hr. Perimeter wells were randomly filled with a different product while each center well was filled with sterile distilled water. Twenty replications of each product were tested. Products evaluated included: hydrogen dioxide (ZeroTol™, BioSafe Systems, Glastonbury, CT; 10 ml/L), quaternary ammonium salts (Physan 20™, Maril Products, Inc., Tustin, CA; 7.5 ml/gal), copper sulfate pentahydrate (Phyton 27®, Source Technology Biologicals, Inc., Edina, MN; 2 fl.oz./10 gal), copper hydroxide + mancozeb (Junction® Griffin, L.L.C., Valdosta, GA; 1.5 lb/100 gal) and two formulations of copper hydroxide (Kocide 101® [1 lb/100 gal] and Kocide 2000® [0.75 lb/100 gal], Griffin, L.L.C., Valdosta, GA).

Plates were observed for zones of inhibition around each well. If bacterial growth suppression occurred, the diameter of the zone of inhibition, minus the diameter of the well, was recorded.

Greenhouse bactericide/disinfectant trial. Tissue culture plugs of *Hosta* x 'Suzanne' (Agri-Starts, Inc., Apopka, FL) produced in 72-cell flats were potted into 0.35 L sterile plastic containers filled with Premier Pro-Mix and placed in the greenhouse under 50 percent shade. Plants were divided into six treatments of 30 plants each. A suspension of *E. carotovora* subsp. *carotovora* (10^8 CFU/ml) was prepared by flooding 24-hr-old cultures grown on NYDA at 30°C with SDW. Using a chromatographic sprayer, bacterial suspensions were applied until runoff (~2 ml/plant). SDW-sprayed plants served as a control.

Plants were sprayed to run-off 24 hr after inoculation with one of the following bactericides/disinfectants: hydroden dioxide (ZeroTol™; 10 ml/L), quaternary ammonium salts (Physan 20™ ; 7.5 ml/gal), copper sulfate pentahydrate (Phyton 27®; 2 fl. oz./10 gal), copper hydroxide (Kocide 101®; 1 lb/100 gal), or copper hydroxide (Kocide 2000®; 0.75 lb/100 gal).

All plants were chilled at 0°C for 24 hr in a Percival cooler 72 hr after chemical were applied. After cold storage, plants were placed in the greenhouse under shade with day and night temperatures of 27°C and 18°C, respectively. Plants were observed for 2 wk and rated using the previously described disease severity scale. The experiment was repeated and data were analyzed by GLM-procedure using SAS statistical software (SAS version 6.12, SAS Institute, Inc., Cary, NC). Means were compared by Tukey's Studentized Range (HSD) Test.

RESULTS

Spread of *E. carotovora* subsp. *carotovora* in cold storage. There was no statistical difference between repeated tests (P= 0.3277). *E. carotovora* subsp. *carotovora* is spread during cold storage of dormant rhizomes(P=0.0001). Sixty to 100 percent of the wounded rhizomes exposed to infected plant material per replication became diseased following cold storage. On average, plants exposed to an infected rhizome during cold storage exhibited severe root decay and limited foliage growth (disease rating of 4) after 2 wk in the greenhouse (data not shown). Only three uninoculated plants out of 50 exhibited some localized decay at the point of division (disease rating of 2).

Spread of *E. carotovora* subsp. *carotovora* by vegetative propagation. There was no statistical difference between repeated tests (P=0.4045). *E. carotovora* subsp. *carotovora* is spread by vegetative propagation of dormant rhizomes. Vegetative propagation using an *E. carotovora* subsp. *carotovora* -inoculated knife transferred *Erwinia* to 60 to 100 percent of hosta rhizomes within each series. The first three sequential divisions exhibited high percentages of bacterial soft rot. By the fourth and fifth sequential division, bacterial soft rot did not develop. Spread of *E. carotovora* subsp. *carotovora* through division using an uninoculated knife was not evident. (Figure 4.1)

***In vitro* bactericide/disinfectant sensitivity assay.** ZeroTol™ was the most effective chemical *in vitro* against *E. carotovora* subsp. *carotovora* causing an average zone of inhibition of 25.5 mm. The copper hydroxide compounds, Kocide 101® and Kocide 2000®, had average zones of inhibition of 12.52 mm and 9.4 mm, respectively. Junction®, another copper-based chemical had a mean of 5.67 mm. Physan 20™, Phyton

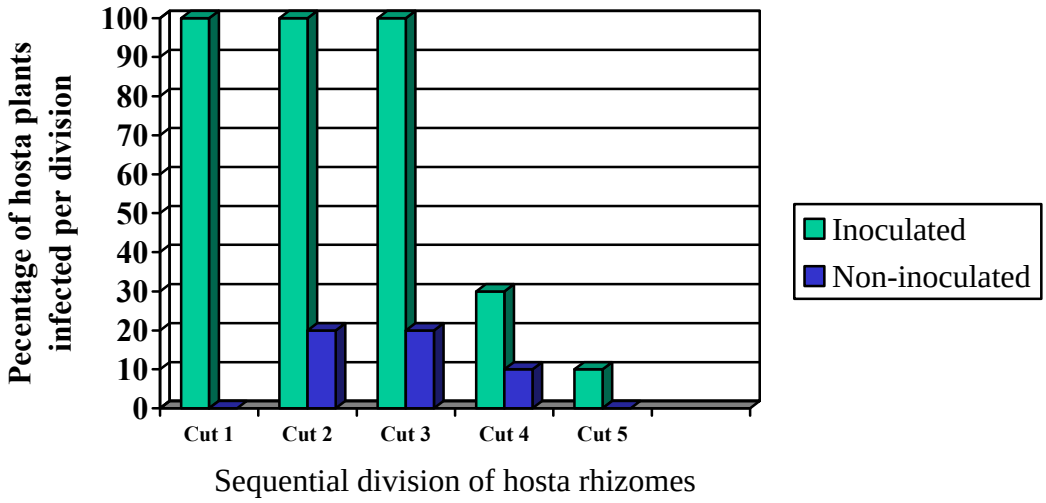

Figure 4.1. Mean bacterial soft rot disease incidence of hosta rhizomes divided sequentially using an *Erwinia carotovora* subsp. *carotovora*-inoculated or sterile knife.

27® and SDW had no effect on the growth of *E. carotovora* subsp. *carotovora*. (Table 4.1)

Greenhouse bactericide/disinfectant trial. Bactericide/disinfectant treatments differed significantly in disease management (*P*=0.0001). None were completely effective at controlling bacterial soft rot development. Phyton 27® treatments resulted in significantly less disease compared to other chemical treatments, and reduced disease development by 50 percent compared to the untreated control. Ninety-seven percent of plants in the SDW treatment developed bacterial soft rot. The copper hydroxide compounds, Kocide 101® and Kocide 2000®, reduced disease incidence, but 67 to 73 percent of the plants, respectively, developed soft rot. Zerotol™ and Physan 20™ did not manage bacterial soft rot and almost all plants were diseased. Most diseased plants in all treatments had advanced soft rot ratings of 4 (most roots decayed and limited foliage growth), 5 (water-soaked leaves and severe root decay), and 6 (complete death). (Table 4.2)

DISCUSSION

Plant material, contaminated tools and insects can harbor *Erwinia carotovora* subsp. *carotovora* (14). *E. carotovora* subsp. *carotovora* is often an epiphyte, residing on foliage and maintaining low populations, becoming pathogenic only after the plant is compromised by another disease or environmental factor (14). It can be present throughout the production of a susceptible host without causing disease (8). *Erwinia* species, including *E. carotovora* subsp. *carotovora* , can be recovered from irrigation water and symptomless hosta foliage in commercial container nurseries (13).

Table 4.1. Average zone of inhibition induced by bactericides and disinfectants on the *in vitro* growth of *Erwinia carotovora* subsp.*carotovora*

Chemical treatment	**Rate**	**Zone of inhibition (mm)[a]**
ZeroTol™	10 ml/L	25.50
Kocide 101®	1 lb/100 gal	12.52
Kocide 2000®	0.75 lb/100 gal	9.40
Junction®	1.5 lb/100 gal	5.67
Phyton 27®	2 fl.oz./10 gal	0.00
Physan 20™	7.5 ml/gal	0.00
Water	n/a	0.00

[a] Diameter of area with no bacterial colony growth minus the diameter of the well (7-mm).

Table 4.2. Percentage of infected plants and average disease severity of spray-inoculated hostas treated with bacteriocides/disinfectants.

Chemical Treatment	Rate	% infected	Disease severity[a]
Phyton 27®	2 fl.oz/10 gal	46.7	2.52 a
Kocide 101®	1 lb/100 gal	66.7	3.60 b
Kocide 2000®	0.75 lb/100 gal	73.3	3.81 b
ZeroTol™	10 ml/liter	95.0	4.95 c
Water	n/a	96.7	5.03 c
Physan 20™	7.5ml/gal	98.3	5.06 c

[a]Average disease severity rated using a 1 to 6 disease rating system where 1=no disease; 2=localized decay, plant overcame; 3=some root decay, but plant is growing; 4=most roots decayed, limited foliage growth; 5=water-soaked leaves, foliage collapses at crown, root decay; 6=complete death, plant does not emerge.

Hosta cultivars are maintained by vegetative propagation, which involves dividing fleshy rhizomes into smaller pieces using hands or a propagation knife (7), thereby wounding the tissue.

If rhizomes are planted in contaminated soil before periderm develops over wounds, plants may become infected with *E. carotovora* subsp. *carotovora* (2). The effect of wounding in bacterial soft rot disease development has been demonstrated in potatoes (*Solanum tuberosum* L.). Injured potato tubers surface-inoculated with *Erwinia carotovora* subsp. *atroseptica* developed soft rot within two days while intact tubers were not infected(16).

Based on this study, it is possible to spread *E. carotovora* subsp. *carotovora* from a primary infected plant to healthy plants in cold storage. The division wound and possible chilling injury provides entry for the pathogen and encourages bacterial growth (3). Although less space is used when hosta rhizomes are stored collectively in bags, it increases the chance of disease spread by direct contact. Elphinstone and Perombelon (4) found that one *E. carotovora* subsp. *carotovora* -infected potato tuber can infect 100 kg of healthy potatoes by direct contact during commercial grading operations. Hosta rhizomes chilled with a single diseased rhizome yielded similar results, with 60 percent or more rhizomes per replication becoming infected.

Sterile media, such as potting soil or peat moss, prolongs the survival of *Erwinia* species (8). The addition of moist peat moss (to prevent the rhizomes from drying) could enable bacteria to endure low temperatures. Although spread of the pathogen occurred in cold storage, disease development occurred after the rhizomes were transferred to the

greenhouse environment. In plant tissue, development of *E. carotovora* subsp. *carotovora* is encouraged by warm temperatures, consistent soil moisture, and high humidity (14).

Plants that were divided using an *E. carotovora* subsp. *carotovora* -inoculated propagation knife were placed immediately into an high-moisture, warm temperature environment. After dividing the infected rhizome, each sequential rhizome had actively growing *E. carotovora* subsp. *carotovora* cells directly applied to a fresh wound. Although transfer of *Erwinia* via pruning tools has been described (11), direct transfer of *E. carotovora* subsp. *carotovora* to herbaceous perennial divisions was thought to occur but was not documented. It is possible to spread bacterial soft rot using an *E. carotovora* subsp. *carotovora* -contaminated knife.

Proper sanitation, including discarding diseased plants, disinfesting knives and other nursery tools can prevent the spread of pathogens within nurseries and greenhouses (9). In potato, inoculated rhizomes that were allowed to air dry for 4 hours prior to planting exhibited lower incidence of *Erwinia* soft rot (2). Although not tested, if injured rhizomes were allowed to dry and form a wound periderm prior to being potted or stored, disease transmission and development may not occur.

Bactericides and disinfectants are ineffective in preventing bacterial soft rot in hosta. Although Phyton 27® exhibited the greatest efficacy of the products evaluated, 47 percent of treated plants developed disease within 72-hours. Other bactericides and disinfectants were even less capable in reducing disease. Epiphytic populations of *E. carotovora* subsp. *carotovora* tend to congregate within petioles and in the crown of the plant (14), therefore, complete chemical coverage may not be possible. Low concentrations of *E. carotovora* subsp. *carotovora* can rapidly increase population

densities and initiate disease when conditions are favorable (3). The chilling injury that occurs when non-dormant plants are held at 0ºC allows entry of the pathogen and subsequent disease.

The inability of Phyton 27® to inhibit bacterial growth *in vitro* may be due to poor diffusion of the product into the surrounding agar medium. *In vitro* assays often show quicker chemical responses, but they may not adequately correspond to product performance *in vivo*. The disinfectant ZeroTol was the most effective at reducing bacterial growth *in vitro*. However, ZeroTol was ineffective at controlling bacterial soft rot with 95% of the inoculated hosta plants.

Erwinia carotovora subsp. *carotovora* is spread through propagative division and disease development is facilitated by injury. This study demonstrated that bacterial soft rot occurs if wounded plants are exposed to *E. carotovora* subsp. *carotovora*, regardless of whether plants were immediately placed in the greenhouse or stored at 0°C. Also, plants with epiphytic populations of *E. carotovora* subsp. *carotovora* can become infected if chilling injury occurs and none of the chemicals tested were satisfactory in managing bacterial soft rot. *Erwinia* spp. are endemic to water and soil and can be present throughout production cycles, therefore nurseries should depend on proper cultural practices to prevent spread and development of bacterial soft rot on hosta.

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CHAPTER 5

DETECTION OF *ERWINIA CAROTOVORA* SUBSP. *CAROTOVORA* IN HOSTA SPP. USING POLYMERASE CHAIN REACTION

Erwinia carotovora subsp. *carotovora* (Jones 1901) Bergey, Harrison, Breed, Hammer & Huntoon 1923 causes a soft rot of many economically important food crops, including potato (*Solanum tuberosum* L.), onion (*Allium cepa* L.), and carrot (*Daucus carota* var. *sativus* L.) (1, 4, 8). It is also a destructive pathogen of many herbaceous ornamental plants, such as hosta (*Hosta* spp. Tratt.), iris (*Iris* spp. L.) and daylily (*Hemerocallis* spp. L.) (2, 9, 11).

The primary symptom of *Erwinia* soft rot is the maceration of fleshy tissue (12). This condition occurs when the pathogen produces pectic enzymes that dissolve the middle lamella of plant cells (12). This separation of cells causes water-soaked lesions to appear on the surface of the infected plant organ. As the disease progresses, parenchyma cell walls are dissolved and the tissue disintegrates. The epidermis of the rhizome or tuber is not affected and will eventually crack to allow the infected tissue to contaminate the surrounding soil (10). Healthy plants that come in contact with either diseased plant material or contaminated soil can become infected (3).

An important aspect of *Erwinia carotovora* subsp. *carotovora* is that it is endemic to soil and water (7) and is commonly isolated from irrigation water, aerosols, rivers and other water sources (1). In addition, it can survive on the foliage of host and non-host

plants as an epiphyte without causing disease. Additionally, *E. carototovora* subsp. *carotovora* has been recovered from healthy plant tissue up to 12 months after inoculation (7).

E. carototovora subsp. *carotovora* is often classified as an opportunistic pathogen because epidemics occur when host tissue is predisposed by other pathogens or mechanical injury (8). Therefore, it can be present without causing disease until the plant is compromised (6).

Recently, bacterial soft rot of hosta caused by *E. carotovora* subsp. *carotovora* was observed at a large wholesale ornamental plant nursery in Berkeley County, South Carolina. In cultivars Aureo-marginata and Golden Tiara, losses of 80 percent or greater occurred. While this disease was previously reported on hosta (5), *E. carotovora* subsp. *carotovora* was not considered a common pathogen of this host. Only after dormant hosta rhizomes were exposed to temperatures of 0°C during artificial chilling did *E. carotovora* subsp. *carotovora* become an important disease of hosta. Wounding due to propagative division of the rhizome coupled with freezing injury allowed *E. carotovora* subsp. *carotovora* to enter the rhizomes and initiate soft rot.

Chemical control of this disease has proven to be ineffective (Chapter IV), therefore *Erwinia* soft rot of hosta must be controlled by exclusion sanitation and proper cultural practices (12). This includes the removal of infected plants before propagation. This can prevent spread of the pathogen to healthy plants. A quick, reliable method for detecting *E. carotovora* subsp. *carotovora* would allow nurseries and tissue-culture labs to identify and remove infected plants before epidemics occur.

Polymerase chain reaction (PCR) is a technique that can be used for detection of specific organisms. It involves duplicating small fragments of deoxyribonucleic acid (DNA) to produce multiple DNA copies. Using specific oligonucleotide primers consisting of several nucleotides, replication of DNA is initiated. Denaturation during the first phase of thermal cycling causes the individual stands of DNA to separate. During the annealing phase, the temperature is lowered to allow the oligonucleotide primers to bind to the DNA. The primers will only react with the target DNA. Finally, the mixture is polymerized to allow replication of DNA. The results of PCR are determined by electrophoresis on an agarose gel and visualized using ultraviolet illumination. PCR allows organisms to be accurately detected at low concentrations. Therefore, this technique would be beneficial for detecting *E. carotovora* subsp. *carotovora* in irrigation sources, soil, and infected plant tissue.

The primary objective of this research was to develop a reliable polymerase chain reaction (PCR)-based assay for the detection of *Erwinia carotovora* subsp. *carotovora.*

MATERIALS AND METHODS

DNA amplification and purification for primer design. Bacterial isolates were amplified by PCR and purified from the PCR reactions. The purified DNA was used to develop a specific primer set for the detection of *Erwinia carotovora* subsp. *carotovora.*

Using PCR, DNA from *Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *atroseptica*, and *Erwinia chrysanthemi* was amplified. One milliliter of a bacterial suspension containing 10^8 CFU/ml of each bacterial isolate was suspended in high pressure liquid chromatography (HPLC) -grade water (J.T. Baker, Phillipsburg, NJ) and boiled for 15 min at 100°C to lyse the bacterial cells and release chromosomal DNA.

PCR reactions were carried out in 25µl mixtures containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 µM of each nucleotide (dATP, dCTP, dGTP, and dTTP), 0.25 µM of each primer and 1 U of *Taq* DNA polymerase (Promega Corp., Madison, WI). Conserved primers EC4 and GS7 (6) were used for DNA amplification. Using a Mastercycler® gradient thermal cycler (Eppendorf Scientific, Hamburg, Germany), amplification of each lysate was conducted. Each reaction tube was incubated in the thermal cycler at 95º C for 5 min, followed by 30 cycles of denaturation at 95° C for 30 s, annealing of primers at 55° C for 30 s, and elongation at 72° C for 30 s. Following the last cycle, amplification continued at 95° C for 30 s, 55° C for 30 s, and ended with a final incubation at 72° C for 5 min. PCR results were determined by electrophoresis (90 V for 45 min in 1x Tris acetate EDTA [TAE] buffer) of 10µl of the PCR product on a 1% agarose gel (50 ml 1 x TAE buffer, 0.5 g high strength analytical grade agarose [Bio-Rad Laboratories, Hercules, CA]) stained with 25µl ethidium bromide (1mg/ml). Following electrophoresis, gels were visualized using ultraviolet transillumination (Eagle Eye II, Stratagene, La Jolla, CA). Using the Wizard® PCR Preps DNA Purification System (Promega Corp., Madison, WI), double-stranded PCR-amplified DNA was purified from the PCR reactions according to the manufacturer's directions.

Developing *Erwinia carotovora* subsp. *carotovora* specific primers. The purified DNA was prepared for sequencing of the 16S-23S internal transcribed sequence region (ITS) for development of a primer set for pathogen detection. The reactions were prepared by combining 4 µl BigDye™ Terminator Ready Reaction Mix (PE Biosystems, Foster City, CA), 16 ng PCR product DNA, 1.6 pmol primer and deionized water, creating 10 µl total

volume mixtures. Cycle sequencing of the PCR mixtures were conducted using a Mastercycler® gradient thermal cycler (Eppendorf Scientific, Hamburg, Germany). The thermal cycler sequencing program was repeated 30 times and consisted of rapid thermal ramp (1º C/sec) to 96º C and held for 10 sec, rapid thermal ramp to 50º C and held for 5 sec, and rapid thermal ramp to 60º C and maintained for 4 min. Following sequencing, the contents of each tube were centrifuged in Dye-X spin columns to purify the sequencing reactions. The reactions were dried down and re-suspended in 15 µl Template Supression Reagent (TSR; Applied Biosystems, Foster City, CA). The suspension was briefly vortexed and then denatured at 95º C for 2 min and chilled on ice for 2 min. The reactions were centrifuged at 14,000 rpm for 1 min. Electrophoresis of each sample was performed using the ABI Prism 210 Genetic Analyzer (Applied Biosystems, Foster City, CA) and DNA nucleotide sequences were generated.

PCR primer selection. Primers were generated by entering the nucleotide sequence into a PCR design program (Center for Genome Research, Whitehall Institute for Biomedial Research, Cambridge, MA). Primers were created from the 16S-23S internal transcribed sequence region from a strain of *Erwinia carotovora* subsp. *carotovora* (*Ecc* 83). The selected primers, labeled ECC K (sense) and W (anti-sense), have sequences of 5'-TGT TGA TGC GAT GAG TGA CA-3' and 5'-CAT CAC TCA CAC CAC ATT ACT-3', respectively. Primer ECC K is from nucleotide positions 70 to 89 and ECC W is from 353 to 372. The segment that includes the chosen primer sequences is 303 base-pairs in length. Primers were synthesized at Molecular Genetics Instrumentation Facility (University of Georgia, Athens, GA).

Determination of specificity of primer set. DNA amplification was conducted on 34 bacteria strains, including *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica* and *E. chrysanthemi* (Table 1). Bacterial lysate (2 µl of each) was included in 25 µl PCR reaction mixtures containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 µM of each nucleotide (dATP, dCTP, dGTP, and dTTP), 0.25 µM of each primer and 1 U of *Taq* DNA polymerase (Promega Corp., Madison, WI). DNA was amplified using a Mastercycler® gradient thermal cycler (Eppendorf Scientific, Hamburg, Germany). The thermal cycler program using primers ECC K and W began with an initial warm-up at 95° C for 5 min, followed by 30 cycles of denaturation at 95° C for 30 s, annealing of primers at 56° C for 30 s, and elongation at 72° C for 30 s. Following the last cycle, amplification continued at 95° C for 30 s, 65° C for 30 s and finally incubated at 72° C for 5 min. To ensure proper annealing temperatures in the thermal cycle program, optimization on a gradient between 51.2° C and 60.9° C was conducted. Annealing at 56° C proved to be the optimal temperature for the primers to bind to the target DNA sequences. The results of PCR were determined by electrophoresis and gels were visualized using ultraviolet transillumination. The experiment was repeated twice.

Threshold of detection by PCR. To determine the sensitivity of primers ECC K and W using PCR, 10-fold serial dilutions of the bacterium were generated in HPLC-grade water, ranging from 10^1 to 10^8 CFU/ml. Concentrations of bacterial cells per dilution were estimated by spread-plating 100µl of each suspension onto plates of nutrient yeast dextrose agar (NYDA; 23 g nutrient agar, 5 g yeast, 10 g dextrose, 1000 ml distilled water). PCR was conducted on 2 µl cell lysates from each treatment using primers ECC

K and W. PCR results were verified by electrophoresis on a 1% agarose gel followed by visualization with ultraviolet light. The experiment was repeated five times.

RESULTS

Determination of specificity of primer set. Of the eight *E. carotovora* subsp. *carotovora* strains tested, 100 percent were amplified by primers ECC K and W. The expected 303-bp DNA amplicons were produced. There was no reaction from other *Erwinia* species examined. The primers did not react with any of the other 31 bacteria tested. (Table 5.1) (Figure 5.1)

Threshold of detection by PCR. Using the primers ECC K and W, reliable detection of *Erwinia carotovora* subsp. *carotovora* occurred when prepared bacterial lysates exceeded were 10^7 CFU/ml concentrations or greater (Figure 5.2). Concentrations below 10^7 CFU/ml yielded no reactions.

DISCUSSION

The primers ECC K and W reliably detect *Erwinia carotovora* subsp. *carotovora* without reacting to other bacteria. PCR using these 16S rRNA primers provides a rapid and accurate method for the detection of this pathogen. Additionally, since it is difficult to separate the soft rot-causing *Erwinia* species using standard biochemical tests (13), this method is useful for identification of *Erwinia carotovora* subsp. *carotovora.*

The primers are not sensitive at low bacterial concentrations. Therefore, conducting PCR using these primers will generally not yield amplifications from water sources or leaf washings from healthy plants. While it would be ideal to detect epiphytic populations using this technique, the primers are not sensitive enough to detect populations below 10^7 CFU/ml. Due to the lack of sensitivity, PCR must be performed

Table 5.1. Phytopathogenic bacteria used in polymerase chain reaction (PCR) with the primers ECC K and W

Bacterium	Isolates Tested	Reaction[a]
Erwinia carotovora subsp. *carotovora*	8	8
E. carotovora subsp. *atroseptica*	3	0
E. chrysanthemi	1	0
Pseudomonas corrugata	1	0
P. acidovorans	1	0
P. coronofaciens	1	0
P. areuginosa	1	0
P. marginalis	1	0
P. fluorescens	1	0
P. agglomerans	1	0
P. viridiflava	1	0
P. syringae pv. *lachrymans*	1	0
P. syringae pv. *glycinea*	1	0
P. syringae pv. *phaseolicola*	1	0
P. syringae pv. *tomato*	1	0
P. syringae pv. *syringae*	1	0
Agrobacterium tumefaciens	1	0
A. radiobacter	1	0
Herbaspirillium rubrisubalbicans	1	0
Comomonas testeroni	1	0
Acidovorax avenae subsp. avenae	1	0
A. avenae subsp. *citrulli*	1	0
A. avenae subsp. *cattleyae*	1	0
A. avenae subsp. *konjaci*	1	0
A. facilis	1	0
Xanthomonas nigramaculans	1	0
X. campestris pv. *campestris*	1	0
X. campestris pv. *vesicatoria*	1	0
X. campestris pv. *raphini*	1	0
X. vignicola	1	0
Burkholderia cepacia	1	0
Clavibacter m. subsp. *michiganensis*	1	0
Pantoea anatas	1	0
Ralstonia solanacearum	1	0

[a] Number of bacterial strains tested yielding a 303-base pair DNA fragment with PCR primers ECC K and W.

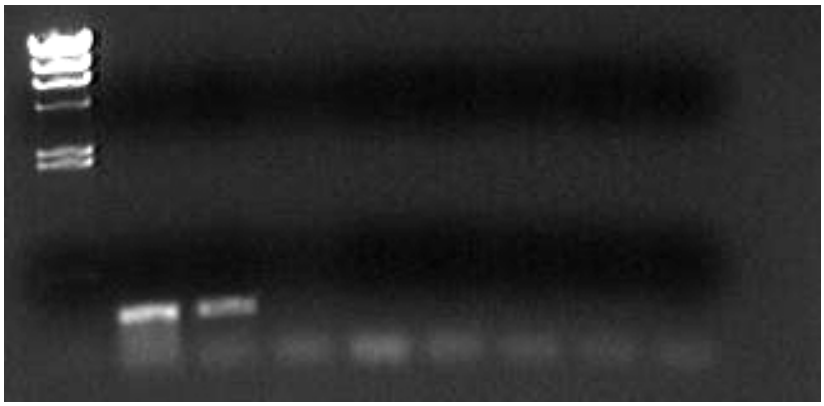

Figure 5.1. Threshold of sensitivity obtained by polymerase chain reaction to detect *Erwinia carotovora* subsp. *carotovora* from cell suspensions containing a range of CFU using primers ECC K and W. Lane M contains a λ marker; lanes 1 to 8 represent PCR on cell suspensions containing 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 CFU/ml.

Table 5.2. Detection of *Erwinia carotovora* subsp. *carotovora* by polymerase chain reaction using primers ECC K and W

Population (CFU/ml)[a]	**Frequency of detection (%)**
10^8	(5/5) 100.00
10^7	(5/5) 100.00
10^6	(0/5) 0.00
10^5	(0/5) 0.00
10^4	(0/5) 0.00
10^3	(0/5) 0.00
10^2	(0/5) 0.00
10^1	(0/5) 0.00
10^0	(0/5) 0.00

[a] *E. carotovora* subsp. *carotovora* cell population.

using artificially-grown bacterial colonies or infected plant material. In soft-rotted plants, pathogenic bacterial concentrations are often greater than the required threshold of these primers. Efforts must be made to improve the sensitivity of the PCR protocol for *E. carotovora* subsp. *carotovora.*

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CHAPTER 6

CONCLUSIONS

In southern climates, artificial chilling can provide many benefits, allowing nurseries to grow plants native to temperate climates, like *Hosta* spp. Implementing a period of low-temperature storage increases plant vigor, producing an earlier crop with improved appearance (2,7). While there are many advantages to using this treatment, disease can occur as a result of environmental manipulation (9). Extensive research has been conducted on the prevention of fruit and vegetable diseases in cold storage (6). In contrast, prevention during cold storage has not been examined in herbaceous perennial plants.

Soft rot caused by *Erwinia carotovora* subsp. *carotovora* is not a frequent disease in hosta. Bacterial soft rot in hosta has been reported but species identification was unknown (3). This disease generally occurs sporadically following unseasonably low temperatures when foliar tissue is not hardened off or dormant. Freezing injury provides the infection court for entry of *E. carotovora* subsp. *carotovora* (8).

E. carotovora subsp. *carotovora* is an epiphytic bacterium that survives on leaf phylloplanes of host and non-host plants at low populations (8). This pathogen, which is frequently found in water sources, can be deposited on the leaves by irrigation water or aerosols (10). *E. carotovora* subsp. *carotovora* was retrieved from 53 percent of water and leaf samples collected from the nursery in South Carolina where extensive soft rot in hosta was seen following artificial chilling and storage at 0°C. Bacterial concentrations

were between 10^0 and 10^2 colony forming units per milliliter (CFU/ml). *E. carotovora* subsp. *carotovora* exists on healthy plants for up to 12 months without causing disease (4). Soft rot usually only occurs after the plant has become compromised by previous infection, mechanical wounding, or environmental stresses (1,8).

Investigation of the influence of low temperature storage on soft rot development revealed that freezing temperatures were required for epiphytic populations of *E. carotovora* subsp. *carotovora* to initiate disease. Incidence of disease increases quadratically as inoculum concentration is elevated from10^2 to 10^8 CFU/ml. Soft rot does not occur when temperatures are maintained above 0° C. Previous research has shown that four weeks of cold storage at 4° C provides adequate chilling for hosta (7).

Spread of this disease from infected hosta rhizomes to healthy rhizomes is facilitated by vegetative propagation. The wound created by division allows entry of *E. carotovora* subsp. *carotovora* bacteria. Plants exposed to an infected rhizome in cold storage experienced high incidence of disease compared to control treatments (P=0.0001). The results were similar in plants divided using an *E. carotovora* subsp. *carotovora* -inoculated propagation knife. Most plants that were divided following an infected rhizome developed soft rot (P=0.0001). The percentage of plants that developed soft rot decreased with the number of divisions from the initial “inoculation”.

Chemical control is not effective in managing this disease. In *in vitro* studies, ZeroTol™, a hydrogen dioxide disinfectant, had the most significant effect on the inhibition of *E. carotovora* subsp. *carotovora* forming zones of inhibition of 25.5mm. The copper hydroxide products, Kocide 101® and Kocide 2000®, were also effective at controlling bacterial growth *in vitro*.

In the greenhouse experiment, copper sulfate pentahydrate (Phyton 27®) provided the most control of bacterial soft rot. Within this treatment, 53 percent of inoculated plants did not develop disease after exposure to freezing temperatures. Following in effectiveness were the copper hydroxide treatments (Kocide 101® and Kocide 2000®), although neither formulation provided sufficient protection. Cultural practices should be relied upon rather than chemical management because none of the treatments in this experiment were satisfactory for nursery production purposes.

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APPENDICES

APPENDIX A

Table A.1. Analysis of variance (ANOVA) of influence of temperature and bacterial concentration on development of *Erwinia carotovora* subsp. *carotovora* in hosta

Source	df	Sum of Squares	F-value	P-value
Test	1	4.17	2.17	0.1786
Conc	3	159.00	27.65	0.0001
Test x Conc	3	3.50	0.61	0.6279
Temp	2	833.33	217.39	0.0001
Conc x Temp	6	318.00	27.65	0.0001
Tests for hypotheses using the Type III MS for Test x Conc as an error term				
Test	1	4.17	3.57	0.1552
Conc	3	159.00	45.43	0.0053

Table A.2. Analysis of variance (ANOVA) of spread of *Erwinia carotovora* subsp. *carotovora* in dormant hosta rhizomes in cold storage

Source	df	Sum of Squares	F-value	P-value
Test	1	1.44	0.97	0.3277
Trt	1	190.44	128.24	0.0001
Test x Trt	1	1.00	0.67	0.4143
Bag(Test x Trt)	16	28.96	1.22	0.2726
Tests of hypotheses using the Type III MS for Test x Trt as an error term				
Trt	1	190.44	190.44	0.0461
Tests of hypotheses using the Type III MS for Bag(Test x Trt) as an error term				
Trt	1	190.44	105.22	0.0001

Table A.3. Analysis of variance (ANOVA) of spread of *Erwinia carotovora* subsp. *carotovora* through sequential propagative division of dormant hosta rhizomes

Source	df	Sum of Squares	F-value	P-value
Test	1	1.44	0.70	0.4045
Trt	1	54.76	26.71	0.0001
Test x Trt	1	0.00	0.00	1.0000
Series(Test x Trt)	16	11.76	0.36	0.9879
Tests of hypotheses using the Type III MS for Series(Test x Trt) as an error term				
Trt	1	54.76	74.50	0.0001

Table A.4. Analysis of variance (ANOVA) of spread of *Erwinia carotovora* subsp. *carotovora* through sequential propagative division of dormant hosta rhizomes in inoculated treatments

Source	df	Sum of Squares	F-value	P-value
Test	1	1.44	0.72	0.3981
Cut	4	47.96	6.00	0.0003
Test x Cut	4	2.76	0.69	0.8466
Tests of hypotheses using the Type III MS for Test x Cut as an error term				
Cut	4	47.96	17.38	0.0086

Table A.5. Analysis of variance (ANOVA) of *Erwinia carotovora* subsp. *carotovora* soft rot severity in hosta plants sprayed with bactericides/disinfectants or non-inoculated

Source	df	Sum of Squares	F-value	P-value
Test	1	35.47	13.81	0.0002
Trt	5	320.45	24.96	0.0001
Test x Trt	5	11.91	2.38	0.4628
Tests of hypotheses using the Type III MS for Test x Trt as an error term				
Trt	5	320.45	64.09	0.0013

Table A.6. Analysis of variance (ANOVA) of *Erwinia carotovora* subsp. *carotovora* soft rot incidence in hosta plants sprayed with bactericides/disinfectants or non-inoculated

Source	df	Sum of Squares	F-value	P-value
Test	1	1.23	9.11	0.0027
Rep	29	4.74	1.22	0.2081
Treatment	5	16.78	24.96	0.0000

APPENDIX B

Table A.7 Table of Bacteriocides/Disinfectants used *in vitro* and in greenhouse trials

Chemical Name	Trade Name	a.i. (%)	Manufacturer	Address
N-alkyl dimethyl benzyl ammonium chloride & N-alkyl dimethyl ethybenzyl ammonium chloride	Physan 20	20	Maril Products, Inc.	Tustin, CA
Copper hydroxide	Kocide 101	72	Griffin, L.L.C.	Valdosta, GA
Copper hydroxide	Kocide 2000	53.8	Griffin, L.L.C.	Valdosta, GA
Copper hydroxide + mancozeb	Junction	45	Griffin, L.L.C.	Valdosta, GA
Hydrogen dioxide	ZeroTol	27	BioSafe Systems	Glastonbury, CT
Copper sulfate pentahydrate	Phyton 27	21.36	Source Technology Biologicals, Inc.	Edina, MN