Development of a systematic chemotherapy assay for the control of blackleg in potato
by Robert Day Davidson

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology
Montana State University

Abstract:
Bacterial diseases of potatoes constitute major problems in the potato industry. Among these, blackleg of potato, caused by Erwinia carotovora var. atroseptica and var. carotovora has been especially severe. Many methods have been proposed for its control and irradica-tion, including chemical seed piece treatment, stem cutting, field roguing, and the use of chemotherapeutic agents. It is the use of chemotherapeutic agents, particularly those with systemic properties, that seems to be gaining momentum at the present time. The utilization of antibiotics as a viable alternative to, or as an enhancement of the other systems currently in practice, is of much interest.

The development of a basic assay model for the use of systemic chemotherapeutic agents on potatoes, particularly for control of economically important diseases such as blackleg, was of primary concern. The assay system worked on included five major steps as listed below: 1) Antibiotics were selected on the basis of known activity, possible plant uptake, and availability on the market.

2) An in-vitro plate bioassay was performed against Erwinia with each antibiotic to be used.

3) An in-vivo plate bioassay of the antibiotic was utilized to determine the proper drug dosage and observe phytotoxicity, if any.

4) All useable antibiotics were checked for systemicity in the plant using a hydroponic system and an in-vitro plate bioassay of the plant parts in question.

5) Upon passing the criteria set by the preceding four steps, plants were subjected to differentially timed antibiotic treatments and challenged by the Erwinia organism to observe the effectiveness of the system.

6) A dye marker was utilized in conjunction with the plant-antibio-tic uptake system to assure proper water uptake and thus antibiotic uptake. Follow-up biochemical tests were performed to be sure of bacterial irradication.

The use of a mist chamber for an easy, practical way to compare the effectiveness of chemical seed piece disinfectants was also discussed. It was found to give reproducible, accurate results and have good potential for implementation into a potato certification program.

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Master of Science

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Plant Pathology

MONTANA STATE UNIVERSITY
Bozeman, Montana

June 1983

APPROVAL

of a thesis submitted by

Robert Day Davidson

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date May 24, 1983

ACKNOWLEDGMENTS

I would like to gratefully acknowledge the many hours of time and guidance that Dr. Dave Sands has provided me in the pursuit of a Master's degree. He has been instrumental in a very positive learning experience in plant pathology. I would also like to acknowledge the assistance, guidance, and editing given by my committee members, Dr. Don Mathre and Dr. Jack Riesselmann. Appreciation should also be given to the Montana Certified Seed Potato Association and those associated with it in their committment to establishing a viable research effort to improve the quality of seed potatoes grown in Montana.

Acknowledgment must go to Mrs. Louise Greene for her help in bringing together the thesis and the hours of typing spent to finish the project before the deadline. Finishing a thesis from out of state is tough and Louise made it seem easy.

Most importantly, I would like to acknowledge with love the overwhelming committment of my wife, Grace, for this undertaking. Her love and assurance were sorely needed on many occasions and the long hours she spent by herself are difficult to repay. Also, the time spent taking care of our daughter when I was unable to be present means much more than can be written here. Thank you.

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ABSTRACT

Bacterial diseases of potatoes constitute major problems in the potato industry. Among these, blackleg of potato, caused by *Erwinia carotovora* var. *atroseptica* and var. *carotovora* has been especially severe. Many methods have been proposed for its control and irradication, including chemical seed piece treatment, stem cutting, field roguing, and the use of chemotherapeutic agents. It is the use of chemotherapeutic agents, particularly those with systemic properties, that seems to be gaining momentum at the present time. The utilization of antibiotics as a viable alternative to, or as an enhancement of the other systems currently in practice, is of much interest.

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CHAPTER 1

INTRODUCTION

Potato blackleg, associated with either of two bacterial soft-rotting pathogens, is a disease that is prevalent wherever potatoes are grown. Disease symptoms include a blackening of the lower stem area on infected plants, an ooze and severe tissue damage near the necrotic site, and, in later stages, leaf yellowing and wilting of the entire plant. The disease is responsible for severe losses in many areas and can cause justifiable removal of a seed potato field from certification. Stand losses and later plant infection resulting in decreased tuber set are common. In storage, tuber losses due to the soft-rot and the water reduction associated with it, as well as, the spread of the pathogen to previously uninfected tubers can become problems in the following year's crop. The disease is most severe when conducive environmental conditions are met. These include periods of high rainfall and/or humidity coupled with cool nights and fairly warm days. Any damage to the crop, such as that associated with hail or machinery, can greatly increase the chances for blackleg occurrence.

The blackleg pathogens, *Erwinia carotovora* var. *atroseptica* (Van Hall) Dye and *E. carotovora* var. *carotovora* (Jones) Dye, abbreviated as Eca and Ecc respectively, are tuber borne and soil borne, with attendant control very difficult. Most current control measures incorporate crop rotation for at least three years to eliminate the organism from the soil, the use of chemical agents such as formaldehyde, mercury or protective chemotherapeutic compounds as tuber disinfectants, roguing infected plants, and the growth of pathogen-free mother plants. A program of culturing the mother plant meristems to avoid the lower stem area where the bacteria are most prevalent, rooting and subsequent placement in the field may also be performed.

The goal of this research was to develop a system of studying systemic antibiotics that potentially could be used as a chemotherapeutic control measure for blackleg disease in the potato's vascular tissue. The system was approached in a way that would be applicable for use by certified potato growers as an integral part of their operation, either by enhancing existing techniques (stem cutting), or as a prophylactic measure for their seed plots. Thus, the main objectives of the research were as follows:

1) to select antibiotics through comprehensive research for determination of their effectiveness against Eca and Ecc and for their performance in an in-vitro plate bioassay.
2) to develop an in-vivo plant assay for determination of both plant reaction to the phytotoxic antibiotics and the maximum non-phytotoxic dosage levels for potato.
3) to ascertain systemicity of compounds in the plant utilizing a hydroponic system and an in-vitro plate bioassay of selected plant parts.
4) to utilize differential, timed antibiotic treatments of potato and subsequent challenge of the plants with _Erwinia_ to observe the system's effectiveness.

5) to use a dye marker in the antibiotic suspension as a verification that general water and chemical transport has occurred.
6) to analyze the use of a mist chamber system for its effectiveness in comparing tuber disinfectants against the blackleg disease.

This methodology could be integrated into a grower's certified seed potato operation in a number of ways. These techniques could be applied during the stem cutting procedure as a protective dip of the stem cuttings prior to rooting or just after rooting, or as a rooted plant dip into the antibiotic during the summer growth of the mother plants. Plants would be replanted in the field to grow the remainder of the season. Either of these applications may help assure blackleg-free plants for use in future crops, especially if integrated into well-managed programs.

CHAPTER 2

LITERATURE REVIEW

Blackleg of potato (*Solanum tuberosum* L.) caused by *Erwinia carotovora* var. *atroseptica* and *E. carotovora* var. *carotovora* has been a major problem in potato crops since their inception as a food source. Economic losses caused by this disease amount to millions of dollars every year (79) and the world-wide distribution makes it a concern or at least a potential threat in most potato producing areas. The bacteria are known to be seed-piece borne (60) and weed and soil borne (43,44), but the main controls of the disease and spread of the pathogen are focused toward control of the seed-piece inoculum (71). Control measures are varied and will be discussed in more detail later. A major problem with the bacterium is that it can enter into the plant and tuber and become systemic where none of the more conventional controls exert any effect (114, 121). These aspects, more than any others, may promote the widespread uses of systemic chemotherapy in the future of the potato industry.

Certified Seed Potato Programs

Certified seed potatoes are grown in almost every state in the U.S. (5). Their importance to the potato industry as a whole is immense. Without strong certified seed programs, the potato industry would be plagued with severe disease problems and probable reductions in potato quality. The overriding concern of all certified seed programs is to produce seed of desirable cultivars that are as disease-free as possible (102). When dealing with topics pertaining to certified seed and the formal structure of the programs, it is important to keep the disease prevention aspects at the forefront. To truly understand the makeup of a certified seed program, it is necessary to take an in-depth look at the principle constituents of a seed plot.

Seed Plots -- Origin of Quality Seed

Most managers of certified seed programs feel that clean disease-free seed starts at the seed plot level (11). This entails the use of small, manageable plots of potatoes which can be tested and checked throughout the season to assure the highest quality possible. Seed plots can be small due to the reproductive ability of the potato. In most cultivars, each hill of potatoes will tend to set tubers at an average rate of three to four per stem or about ten per hill. From each hill it is possible to harvest the tubers, store, and, the following season, cut each tuber into four pieces which permits the planting of approximately forty new hills versus the one hill of the previous year. Thus, major increases in production can be implemented while the original seed plot is kept to a minimum size (124).

Usually the plot consists of plants individually selected for quality characteristics, including yield and tuber size. The plot is harvested by hand and the tubers are sacked individually for storage. The seed plot is isolated from other fields (the distance varies from grower to grower, but usually is not less than 20 feet) to reduce the chance for disease infestation and the harvested seed is kept isolated from all other seed stocks to assure continuity and to avoid disease cross contamination. The tubers used as seed in the plot may be the progeny of mother plants either field grown or greenhouse grown the previous year. These are plants which are line selected, i.e., one plant or group of agronomically uniform plants are grown through successive years thus perpetuating the genetic and phenotypic continuity of a single clone (11). They may also be from stem cut tubers (32) which have been grown in the greenhouse from which stem cuttings were taken. They are then transplanted to the field to be grown, harvested, and stored for the following year's use in the seed plot. These tubers are again hand selected, harvested, individually bagged, and stored in isolation.

Seed plots are planted with a double tuber unit's worth of space between each tuber unit or hill unit. A tuber unit is one in which the identity of the tuber is maintained through the cutting operation with all of the pieces from the tuber planted as an individual unit. Hill units, on the other hand, permit identity of all of the tubers taken from the mother plant the previous year as these are cut and planted together as a unit. This system of hill or tuber unit planting allows for individual testing of each unit for the presence of many different pathogens. These include the presence of viruses, mainly Potato Virus X and Y (PVX and PVY) and Potato Leaf Roll Virus (PLRV), the presence of bacterial pathogens such as <u>Erwinia</u> and <u>Corynebacterium sepedonicum</u>, the presence of fungal pathogens such as <u>Fusarium</u>, <u>Verticillium</u> and <u>Alternaria</u>, and various nematodes. Also, the overall quality of the crop is closely observed (11). Many of these tests are accomplished by visual means through field inspections; but many plants are also examined utilizing testing programs such as the PVX summer testing laboratory (123) and, more recently, the utilization of the ELISA technique for detection of potato viruses and bacteria in the tuber (30). These tests are run by the certifying agency.

Upon completion of the testing and the removal of all infected or suspect plants, the plot is visually inspected for diseases during the remainder of its growth. Disinfection of equipment and sanitation of personnel entering the field is crucial to minimize pathogen spread. Signs and dip pans (pans filled with disinfectants for the dipping of shoes) are kept at all entrances to the plot and intensive field roguing is performed during the season. Accepted practice dictates that either the seed plot is the first planted and the first harvested to insure proper equipment sanitation, or separate equipment is utilized.

All of the above steps are necessary to insure the highest quality, cleanest seed possible. Thus, because of the grower's care and the limited nature of the seed plot's size, many things can be done to the potatoes that would not ordinarily be performed in larger fields. Introduction of new techniques is possible, such as, systemic chemotherapy and the laboratory use of the mist chamber. Major concerns about the toxicity of the chemicals can be reduced at this level as this seed is never allowed to enter into the commercial potato market (11). These procedures, being introduced with the seed plot, insure the most cost productive use of time, chemicals, and land, as well as, the most manageable program for the average grower. This program will enhance the economic stability of the crop while increasing quality and productivity. The grower needs economic leverage in order to sell his seed crop at a profit, and the use of systemic chemotherapy can insure a cleaner and thus higher value seed. As quality of the seed rises, especially where disease-free status is observed, it sets the basis for commanding a higher price on the seed market (11). The seed business is a high risk venture, with commensurate high rewards.

Beyond the Seed Plot

Future crops grown from the potatoes derived from the seed plot can, at best, only be equal in quality to the seed used. If all necessary measures have been observed at the seed plot level, then as the acreage is increased, seed handling, field roguing, and harvest become much less costly. Disease incidence increases cause a disproportionate increase in time spent correcting the problem. Thus, the importance of clean seed is generally only perceived when a problem with the seed arises (124).

The Blackleg Disease

Blackleg has become a disease of world-wide concern, because of its destructive ability (yield and storage losses) and the ease with which its uncontrollable nature allows it to occur from year to year (71). Two varieties of the bacterial species, *Erwinia carotovora*, are the causal organisms. The abbreviations Eca and Ecc, as discussed in the Introduction, will be used throughout this thesis. Both varieties are able to infect potato and cause the classic blackleg disease symptoms. Under the proper conditions either may infect the plant or tuber. Eca has not only been shown to infect potato, but sunflower and sugarbeets as well (71). Ecc is known to infect many additional species of plants, including most families of fruits and vegetables (114). Economic losses from Eca and Ecc may be very extensive, with over 16 states reporting these organisms as their number one causal agent for disease loss in 1980. This ranks a close second behind E. amylovora on the list of most important bacterial pathogens in the most states and ranks sixth overall in the U.S. for causing economic loss on all crops (79).

Disease Symptoms

Any stage of the potato plant is susceptible to the blackleg pathogen. These include pre- and post-emergence of the shoots, seed pieces before and after shoot formation, leaves and petioles on the growing plants, and daughter tubers before harvest or during their subsequent storage (71). Symptoms are characterized by a blackened, necrotic area near the infection site on the seed piece or stem, hence the name blackleg. An accompanying soft rot of the infected areas may also be present. Bacterial ooze, creamy to clear in color, may appear at any stage of symptom expression. It is possible to observe wilt of the infected stem. Death of the plant often occurs in the later stages. A characteristic strong odor is usually present when symptoms are visible.

The infection generally originates from the seed piece and then extends up the stem for a few centimeters or for its entire length. Vascular discoloration is evident above the blackened area and the plant may show a stunted, stiff erect type of growth. The leaves tend to roll upward at the margins and may become chlorotic in the later stages of the disease. The stem may also become infected in relation to damage from wind (118), hail, or machinery (114). It may show typical blackleg symptoms even though the seed piece is sound. The bacteria can be found in most seed stocks as shown by Perombelon (109) and may remain latent (69,75) causing an outbreak of disease only when proper environmental conditions occur. These studies alone indicate the need for a program as encompassing as systemic chemotherapy.

The most common means of daughter tuber infection is bacterial movement along the stolon from the seed piece or infected stem, thus most infected tubers show a black rotted area around the stolon end (43). Infection may also occur through lenticles (1), wounds (54), stem scars or petioles on the plant (114), or lenticles and wounds on the tuber (86). Soft-rot symptoms include vascular discoloration and tuber rot. The decay is clearly delineated from the healthy tissue and, when washed with water, the infected tissue sluffs off and the intact, healthy tissue remains (71). Bacterial ooze may be present inside the tuber, but there is generally no odor associated with this infection. In its later stages, a distinctive strong odor may result because of secondary invasion by *Clostridium*, *Pseudomonas* and/or *Bacillus* (87).

Causal Organisms

E. carotovora are Gram negative bacteria with a rod shape and peritrichous flagella. They are about 0.7 x 1.54 mm in size, are non-spore forming, and are facultatively anaerobic (25). *E. chrysanthemi* should be included in the group for its involvement in some blackleg-like diseases in Peru (77). These organisms are easily cultured. The usual media contain sodium polypectate (polygalacturonic acid). The bacteria form deep pits or wells in the medium as a result of pectin hydrolysis. The best known media for blackleg isolation and growth are Cuppels' and Kelman's Crystal Violet Pectinate (CVP) (39), Stewart MacConkey's medium

(133), and Miller and Schroth's Brom Thymol Blue medium (94). Anaerobic incubation of the medium, followed by direct observation of colony morphology, results in exclusion of the majority of contaminants.

Table 1. Differentiations between the two varieties of E. carotovora and E. chrysanthemi (114).

Test	Ecc	Eca	E. chrysanthemi
Sensitivity to erythromycin (50 g/ml)	-	-	-
Reducing substance from sucrose	-	+	+
Phosphatase	-	-	+
Gas from glucose	-	-	+
Pectin degradation	+	+	+
Gelatin liquification	+	+	V*
Potato soft rot	+	+	+
Acid from: lactose	+	+	V*
-methylglucoside	-	+	-
palatinose	-	+	-

*V = Variable

Disease Cycle

The disease cycle begins when an infected seed piece is planted. The bacteria may reside in or on the potato seed piece. They are spread to other seed pieces during the cutting process, planting and cultivation, and by small flies and other insects. At this time, pre-emergence blackleg may take effect and cause stand losses and/or destroy primary sprouts. Infected plants which do grow to produce daughter tubers may help spread the bacteria to these tubers. Even if the plant does not survive, the bacteria may be spread to other tuber surfaces during the season or harvest (67). After harvest and storage, the organisms overwinter in the tuber pile. The amount of infection in the field will often dictate the amount of spread in the storage unit. Tuber breakdown may be hastened by high respiration or increased CO_2 levels (143). Excessive condensation on the tuber surface during storage is particularly harmful, as it may set up a moisture film on the outside resulting in an effect similar to that artificially obtained in a mist chamber, where oxygen potential is reduced and the rotting potential is dramatically increased (87). Spread of the bacteria to proximal tubers in the tuber pile often occurs. Any infected tubers are then carried on as the following year's mother tubers allowing for disease spread during the planting and growing season as previously described. The bacteria may overwinter in the soil, but only under very unusual circumstances which include predisposing temperature and moisture conditions (114).

Epidemiology

Eca is spread to daughter tubers usually under moist soil conditions and fairly cool soil temperatures (18-20°C). It is more of a problem in areas where these conditions exist for long periods of time, as in the northern regions (71). Survival of Eca in the soil varies with the soil conditions. When the bacteria do not originate in the infected seed piece, their subsequent survival is greatly affected by soil temperature and moisture. Molina and Harrison (99) found that the cells may survive for 80-110 days at 2°C, but as the temperature became higher, the survival time decreased sharply. Warm and dry soil conditions not only favor a low blackleg incidence, but also tend to reduce bacterial migration in the soil from tuber to tuber (110). On the other hand, cool temperatures and wet soils coupled with high daytime temperatures during or after plant emergence and growth tend to predispose the potato to post-emergence blackleg infection (4). Thus, since high temperatures favor a decay of the seed piece and also can cause greater pre-emergence damage, the losses attributed to blackleg are usually higher in the warmer climates (71). This may also favor infection by Ecc, especially when soil temperatures approach the 30-35^{o}C range. It appears that survival of the pathogen may be better at cooler temperatures, but infection may be better in the warmer temperatures.

Spread of the bacteria in other ways also occurs. Several insect species are known vectors, spreading the pathogen from cull piles and dumps to clean fields (66). Graham in England (62) showed that aerosols created by rain and/or sprinkler irrigation systems can disseminate the blackleg pathogen and that mechanical vine destruction, as well as seed piece cutting operations, can also enhance its spread. In Arizona and Idaho, seed piece rot by Fusarium can predispose the potato tuber to invasion by both Eca and Ecc and may result in more tubers showing blackleg, even when conditions are otherwise not favorable for disease expression (104).

Ecc is spread in a slightly different manner than Eca due to variations in temperature response and organism characteristics. Soft-rot development is influenced by several factors. Immature tubers show a greater infection rate than do mature tubers as they have thin skins and greater predisposition to infection (71). Wounds, solar irradiation (103), and high temperature coupled with high soil moisture content which tends to reduce the oxygen potential and thus increase the chance for infection (143) are also factors. Harvesting of seed potatoes above a soil temperature of 20-25°C may enhance infection of the next generation, especially if bruising occurs (113). The optimum range of temperature for soft-rot decay is 25-30°C and is also the best growing range for Ecc in-vitro (71). Conditions associated with high nitrogen fertilization, poor soil aeration, flood irrigation, and the presence of a water film over the tuber after washing can favor development of soft rot (87).

In summary, both Eca and Ecc are favorably influenced by soil temperatures in the range of 18-22°C for the former and 20-30°C for the latter, by high soil moisture, and by the stage of the potato's growth. Infection by Eca is optimum during cool temperatures and moist soil conditions causing seed piece decay, pre- and post-emergence damage, and tuber rots. Ecc, on the other hand, shows optimum infection at slightly higher temperatures (30-35°C) while resulting in plant responses similar to those brought about by Eca. Either pathogen may be the causal organism of blackleg symptoms in the field, but in many cases, disease spread and infection can be circumvented by proper handling of the tubers during harvest and storage.

Current Control Methods

Disease control methods are reviewed in the Compendium of Potato Diseases (71). Control of Eca within a certified seed program includes the following: plant certified seed, free of the blackleg pathogen; plant in well-drained soil; avoid excessive irrigation; treat the seed with registered fungicides or allow cut seed to suberize properly to avoid contamination with other pathogens such as *Fusarium*, which may predispose the seed to blackleg infection; utilize stem cut seed if available; plant in fields which have been out of potato production for at least three years to avoid recontamination of healthy stocks; remove potato cull piles, refuse dumps, or any other inoculation source which may allow insect transmission of the pathogen to the field; do not wash the seed if possible and handle in such a way that bruising and wounds are kept to a minimum; maintain a balanced fertilizer program to prevent plant stress; rogue and carry from the field all infected plants and tubers and their neighbors, as they appear, to reduce the chance of spread to healthy plants.

Control for Ecc includes the following procedures in addition to the steps given for Eca: Lenticle infection may be reduced by harvesting only when soil moisture is low; tubers should be harvested only when they have reached maturity and then only when soil temperatures are less than a daily maximum of 20°C; maintain proper care and handling of the tubers during harvest to keep wounds and tuber damage to a minimum; keep tubers from being exposed to solar irradiation and excessive drying; store tubers at cool temperatures (1.6-4.5°C or about 38-42°F) and be sure they are cooled as rapidly as possible to the storage temperature; proper curing of the tubers is essential; keep water films and CO_2 from accumulating by proper regulation of storage air flow fans and temperature control; condensation may develop from high humidity in storage and a very low pulp temperature in the potato; do not wash tubers before storage, but if they are washed for shipping, use clean, chlorine disinfected water, dry the tubers rapidly, and keep them well aerated throughout the shipping process.

Many of these controls are applicable in today's seed programs, but there are also many controls that are not totally effective and may only be the lesser of two evils. Surface disinfection of the tubers is one such example. Even though all bacteria may be killed on the surface and in the lenticles, unless the bacteria within the tuber are reached, blackleg can still manifest itself in the crop. As bacteria within the tuber cannot be eliminated by conventional methodology (11), development and use of systemic chemotherapeutants may be in order.

History of Plant Protection with Antibiotics

Chemotherapeutic treatment of bacterial plant diseases has been used in agriculture for the last 30-40 years. The years following the discovery of penicillin in 1929 and streptomycin in 1944 by Waksman et al. (139) were the period of peak research activity for the use of antibiotics on plants and during this time, many of the systemic compounds were discovered. Use of these compounds has been relatively slow in recent years due to many factors. These include the prolonged use of many broad spectrum biocides like the mercurials and formaldehyde. Also, one of the problems with early antibiotics was their inability to meet standard animal health requirements and still show plant pathogen control. Now, however, with suspended use of mercury and the major disadvantages associated with formaldehyde, pyrrolidine and other broad spectrum chemicals, including a disagreeable smell coupled with presumptive health hazards, the use of chemotherapeutic agents may become more widespread.

Several researchers worked with antibiotics and studied their role in plant protection in the early 1950's and 1960's. One of the foremost was Peter Ark. His work with the streptomycin compounds was quite extensive. He demonstrated systemic action in many plants (2,3,9,10), as well as control of many plant pathogens in-vitro and in-vivo (10). He also examined one of the major problems of systemic plant protectants, that of mutability of the pathogen toward antibiotic resistance. He found that streptomycin incited high mutability of the pathogen (57). Later researchers have substantiated these results and have shown that control of most bacterial pathogens is a dose dependent phenomenon (23, 57). Pramer et al. (116) demonstrated for the first time streptomycin inhibition of the pathogen in a direct manner. Using a streptomycin resistant strain of _E. chrysanthemi_, he was able to establish a plant infection and maintain the pathogen in a viable state after an inhibitory concentration of streptomycin was applied. Jeffreys (74) demonstrated that antibiotic stability in soils was very low. Even sand could bind large amounts of streptomycin and penicillin, effectively reducing their concentrations in the soil. Organic compounds were rapidly metabolized by the soil normal flora and absorption by colloids in the soil was found to inactivate many compounds.

Methods of Applying Antibiotics

Methods for applying antibiotics to plants are quite varied. Bonde (14,17,18,19), in the early 1950's, worked extensively with potato tuber treatments using streptomycin and oxytetracycline (Terramycin). He found that they provided very effective control of the blackleg organism, but increased susceptibility to _Fusarium_ seed piece decay. This research stressed the importance of combined chemical treatments dealing with more than one problem at the same time.

Spray treatments, such as those used for the control of the fire-blight pathogen E. *amylovora*, are quite common and very effective (58). Also, injection of antibiotics into stems and trunks has shown some success in the treatment of crown gall tumors, walnut blight, and certain diseases caused by Mycoplasma-Like Organisms (57,116). Systemic chemicals, dusts, and topical applications can also be quite useful. However, there are still many problems associated with the use of antibiotics. Important points concern stability and persistence of the compounds used, compatibility with other agricultural chemicals, effective bioassays, and phytotoxicity. Although all are important, phytotoxicity is probably the most serious.

Phytotoxicity

Many chemicals have demonstrated systemic activity, adequate stability in the tissue, and a high level of compatibility with other chemicals, but, unfortunately, they also have excessive phytotoxicity (57). The antibiotics must show reasonable effectiveness against the pathogen in question, but not damage the treated plant beyond certain limits.

Phytotoxic symptoms, caused by antibiotic application to the plant, include, germination inhibition, root thickening, lack of root growth, stunting of the stem, reduced leaf expansion, and chlorosis of the leaf tissue. Chlorosis and root growth inhibition are the most common toxic effects as listed by Brian (23). Streptomycin induces all of these symptoms to some extent, while oxytetracycline and chloramphenicol tend to cause only decreased root and shoot growth and leaf chlorosis. Several authors have demonstrated phytotoxic effects on many vegetables, most often with streptomycin (23). Marlett outlined the foliar symptoms on many plant species (91). Gray, on the other hand, showed large inhibitions of root growth with streptomycin (63). Reversal of the inhibition was obtained with manganese applications.

Antibiotics affect various metabolic target sites of procaryotic cells, but in eucaryotic cells they tend to affect the chloroplasts, resulting in pigment destruction or chloroplast degradation and thus chlorosis of the leaf tissue (57). While the overall health of the plant is desired, at reasonable levels, temporary reductions in plant health and vigor may be a permissible tradeoff for guaranteeing clean seed.

Factors Affecting Systemic Action

Nelson and Hwang in 1975 (101) explored water usage in potato plants at different growth stages. They found that the most rapid water usage occurred during the young plant stage, from emergence to tuber initiation. After this stage, it remained stable and finally tapered off during senescence and death. This is an important consideration when using systemic compounds, as the young plant stage becomes the most appropriate, effective time for root application. Root barriers can pose a problem in some antibiotic uptake systems (as in the case of streptomycin) (7), so the stage of plant growth at application time, as well as the method of application can become important. Absorption through the roots can be increased by the addition of wetting agents and, in the case of streptomycin, 1% glycerol (64) or K_2HPO_4 (57). Root antibiotic uptake is generally slower than that seen with stem cuttings. Pramer (116) suggests that streptomycin is accumulated against a concentration gradient or that active transport may be necessary. He also showed enzyme inhibitors could reduce streptomycin uptake, providing further evidence for active uptake. Absorption of antibiotics has been demonstrated in seeds, stem tissue, foliar applications, and by root uptake. In the case of potato, a root dip treatment will probably provide the most effective, cost productive method of antibiotic uptake, particularly since acropetal xylem movement is the best explanation to date for antibiotic translocation in the plant. However, some movement through the phloem in a basipetal direction is known to occur and the possibility of using foliar sprays does exist (57).

Antibiotic Mode of Action

The mode of action of an antibiotic on a bacterial cell can be on a single site or on many different metabolic processes (135). In addition to direct action of the drug upon the pathogen, as in the case of streptomycin against Erwinia carotovora (19), antibiotics can have indirect effects. These include the neutralizing of toxins secreted by the pathogen, a chemical conversion of the antibiotic within the plant to a substance having a greater or different action on the pathogen, or a direct action of the compound upon the host; e.g., streptomycin eliciting the host polyphenolase system response resulting in control of late blight of potatoes (136). The antibiotic may also show enhancement within the system with the use of non-antibiotic substances such as glycerol (64) to improve uptake and thus deliver more of the drug to the site of infection. The antibiotic may also eliminate other synergistic or antagonistic bacteria present in the plant.

Development of Resistance

Of the many potential problems that exist with systemic antibiotic use, the most serious concern for the plant pathologist is the pathogen's ability to develop resistance. Early work with antibiotics was based on the germ theory of disease: "Specific infections are caused by specific microbes and a drug is best selected for its ability to destroy them" (137). However, even though a given microbe may be the causal agent of a disease, repeated use of a certain drug for control may result in bacterial resistance to treatment. Within modern medicine, mutability and/or transfer of the R factors for resistance to chemotherapeutic agents are always major concerns (6). Likewise, mutability of plant pathogenic bacteria should be of primary importance to any plant pathologist working with antibiotics. No R factors have been observed in plant pathogenic bacteria from the field, although they can be introduced in the lab into phytopathogenic strains. Nonetheless, as antibiotic use increases, we might expect R factors to appear in nature. In most cases, only high pathogen populations will lead to mutations. If antibiotics are used in otherwise clean seed programs, there is probably less likelihood of resistance developing (57).

R Factors

Resistance to compounds in an organism can occur in two ways. It may be the result of a point mutation or other change in the structure of the genetic information of the bacterium or it may be the exchange of genetic information from one bacterium to another via transduction or conjugation. By far the most important method for spreading resistance is the exchange of genetic information, denoted infectious drug resistance, because sensitive bacteria become infected with the resistance factors. These are able to move from one bacterium to another even though the antibiotic is not present (140). Plasmids are usually responsible for this exchange of information and in Gram negative cells such as Erwinia they are known as R factors.

There are two major types of R factor genes: those carrying the determinants for antibiotic resistance and those carrying the transfer factors to move the plasmid from cell to cell. These are labeled resistance determinant (RD) and resistance transfer factor (RFT) respectively. The number of antibiotics that resistance is built against is determined by the number of RD's attached to a given RTF (6). As many as seven genes for resistance to seven different antibiotics have been found on one R factor. Also, R factors may combine to produce more RD's on one R factor and thus more drug resistance in a given call (96).

There is no one single factor for determining bacterial resistance. The most common mode of resistance is a step-wise series of reductions in the organism's ability to absorb the drug or a corresponding increase in cell permeability which makes it impossible to accumulate enough of the drug to effectively control the bacteria (115). Another factor results when the pathogen develops an enzyme to destroy or inactivate the drug, as in the case of penicillinase which opens the B-lactam ring (21). Development of an alternate enzyme system to metabolize products by an alternate pathway may be of importance, as in the case of the sugar derivative antibiotics like streptomycin. Also, a mutation leading to alteration of the active site in the target organism is common (57).

Resistance by direct organism chromosomal changes is not often seen. The changes usually occur in only one out of every 10^7-10^{12} bacteria and are generally difficult to detect (140). In this regard, streptomycin ranks as the number one problem antibiotic as, only a single step mutation can mask the attachment site on the ribosome and create almost total resistance (139). This can play an important role in disease control if streptomycin is the drug of choice.

Drug Combinations

Combinations are important when dealing with antibiotics for many reasons as outlined by Waksman (139).

1) A more effective bacteriostatic or bacteriocidal action may be obtained.
2) The danger from the development of resistance is reduced.
3) Resistant organisms may be destroyed by using a small fraction of the drug which the organism is resistant to instead of the huge dose which may be required.
4) The toxicity of the drug (in our case phytotoxicity) may be minimized due to the lower concentration used in combination for the same degree of activity.
5) There is the potential for longer treatment times for a more complete control and an improved host response.

All of these can play a role in how well a given drug or drug combination works. When dealing with plants, it can become imperative to utilize combinations. Often a grower may be using a dip treatment and, as he uses it, the compound mixture becomes more and more dilute due to organic matter and binding of the antibiotic, heat, sunlight, and the removal of the active ingredient onto the plant surface. These can reduce the compound's effectiveness. It may even set up a sequence conducive to mutation of the pathogen. Bonde (19) found that he could dip up to 2000 cwt. (sacks) of potatoes in the same Agrimycin (streptomycin) treatment with very little loss in activity; however, the potatoes were washed first. New techniques for developing combinatory studies are now available and can be utilized in programs of this sort (13). By using drugs in combination, the above concerns can be reduced. Also, use of different compounds each growing season provides a scheme where mutations may become less likely. With antibiotics as potential threats to human health, combinatory studies can become extremely useful in that drug dosages can be kept to a minimum.

Formulation of antibiotics also becomes a key factor. For uptake, they should be in a soluble form; that is, not bound to a clay carrier, and they should be buffered as many antibiotics are unstable in alkaline solutions. Similarly additives such as manganese can reduce phytotoxicity and permit higher dosages (63).

CHAPTER 3

BACTERIAL IN-VITRO PLATE ASSAYS

Bacteriocide Selection, Assay for Effectiveness, and Phytotoxicity

The basic premise in developing any assay system for testing antibiotic agents against a micro-organism is that there are compounds which will work effectively against the organism in question. The key goals in this study were: 1) to determine which compounds are effective against the blackleg organism, 2) to test their effectiveness by an in-vitro plate bioassay, and 3) to use this information to incorporate the qualifying compounds into an in-vivo test to observe plants for phytotoxic effects.

Materials and Methods

Selection of the Antimicrobial Agents

A fast, efficient way to check for a compound's antibiotic activity was to survey the literature. Generally included was information on the main groups of compounds, their uses, structures, modes of action, and brief histories. Some references also had information pertaining to possible problems within groups of compounds (such as mutative properties) and their overall relationship with the other groups. The compounds selected showed potential activity against Erwinia. They also were selected from more than one antibiotic group, offsetting the chance for a single mutation by Erwinia around a given compound or group of compounds and consequential loss of disease control.

The antibiotics selected are listed in Table 2 in alphabetical order. Included is a brief summary on their basic activity, mode of action, and the group they belong in. Other general pertinent information is listed in Table 3. (21,57,117). All compounds were obtained from Sigma Biochemicals, St. Louis, Missouri unless otherwise noted.

In-vitro Plate Bioassay for Compound Effectiveness Against Blackleg -- Experimental Design

The method utilized was similar to the Bauer-Kirby single disc zone measurement using Bacto-Mueller Hinton Medium (Difco) (12). Bacteria were grown 72 hours before use on Crystal Violet Pectate (CVP) medium to assure their identity. They were then transferred to Nutrient Agar and allowed to grow for 36 hours before removal for suspension in sterile water. Plates of Mueller Hinton medium were overlayered with Eca and Ecc or both at a rate of 0.1 ml. of 4 x 10^8 cfu/ml. (OD_{640} = 0.1) and allowed to dry for 15 minutes. At this point, white, 12.7 mm., sterile antimicrobial sensitivity testing discs were soaked in given amounts of the antibiotic being tested and placed on the plates in a standardized pattern as dictated in the Difco Lab bulletin #0345 (1980). The plates were then incubated at room temperature (22-24°C) for 48 hours. Subsequent zones of microbial growth inhibition, that is, the distance from the outer edge of the disc to the outer edge of the growth inhibition, were measured in millimeters. Each antibiotic was used in suspension at 1000 μg/ml., 500 μg/ml., and 100 μg/ml. Antibiotic combinations were performed using suspensions of 500 μg/ml. of each. Combinations were rated on the basis of enhancement, lack of change, or reduction in the

Table 2. Summary of antibiotics tested.

Antibiotics	Activity	Mode of Action	Group
albamycin (Novobiocin)	Gram (+) & (-)		Miscellaneous
bacitracin	Gram (+)	Interference with Cell Wall synthesis	The Peptides
Bayer #1-4 (Bayer Co.)			
chloramphenicol	Broad spectrum Gram (+) & (-)	Inhibitory to protein synthesis. Competes with mRNA for ribosomal binding at 50s site.	Chloramphenicol
erythromycin	Gram (+) but resistant to penicillinase	Similar to chloramphenicol	The Macrolides
fluorocytosine	Anti-fungal with some anti-bacterial	Blocks thymidylate synthetase to help prevent nucleic acid synthesis.	
G 418 Sulfate (Schering Co.)		Similar to the aminoglycosides	The Aminoglycosides
J 1-20 (Schering Co.)	No available information -		
kasugamycin	Gram (-)	Similar to aminoglycosides	
naladixic acid	Gram (-) bacilli		
oxytetracycline (Terramycin)	Broad spectrum Gram (+) & (-)	Impaired function of ribosomes by blocking the binding of the tRNA at the 30s unit.	The Tetracyclines
penicillin G	Mainly Gram (+) but some Gram (-)	Inhibits incorporation of amino acids into the bacterial cell wall creating a defective mucopeptide.	The Peptides

Table 2. (Continued)

Antibiotics	Activity	Mode of Action	Group
Rondomycin (methacycline)	Gram (-)	Similar to oxytetracycline.	The Tetracyclines
Sch 15445 (Schering Co.)	No available information -		
streptomycin (Agrimycin)	Broad spectrum Gram (+) & (-), Anti-fungal and Anti-tuberculin.	Blocks the 30s ribosomal unit causing early leaving of the m RNA. Also, interferes with tRNA binding.	The Aminoglycosides
S-2 amino ethyl-L-cysteine hydrochloride	Anti-bacterial		
D-L tartaric acid (500mM at pH-3.0)	Anti-bacterial		
trimethoprim	Anti-bacterial		The Diaminopyrimidines
2, 3-5 triphenyl tetrazolium chloride (TZC)	Included as a dye marker		
tylosine tartrate	Anti-bacterial		The Macrolides

Table 3. General information about some of the antibiotics tested.

Antibiotics	General Information	
albamycin	Discovery--1956	
bacitracin	Discovery--1945	Consists of types A (most active), B, D, E, and F. Useful for the synergistic response with penicillin.
chloramphenicol	Discovery--1947	Derivable from one amino acid. Proven plant systemic.
erythromycin	Discovery--1952	Derived from proprionate or acetate units.
naladixic acid		Used in selective media due to its narrow band of activity.
oxytetracycline	Discovery--1950	Derived from proprionate or acetate units. Low toxicity and a proven plant systemic.
penicillin G	Discovery--1929	Not resistant to penicillinase. Derived from two amino acids and is a proven plant systemic.
streptomycin	Discovery--1944	Derived from sugars. Water soluble with bacteriostatic action in low concentrations and bacteriocidal action in high concentrations. Extreme mutational problems with the target pathogens. Extensively used in agriculture and a proven plant systemic.
D-L tartaric acid		Used in the control of fireblight, E. amylovora (58).
2, 3-5 triphenyl tetrazolium chloride		Used to enhance Gram (-) detection on certain media as a red precipitate forms when this is broken down.

Other compounds which exhibit particularly strong Gram (-) activity include kanamycin, neomycin, and some of the penicillinase resistant penicillins. These were excluded from the experimental design only because of limitations of experimental material and as duplicate representatives of the same groups.

size of the growth inhibition zone as compared with either of the two antibiotics used alone. Three separate plates or replications were performed with each antibiotic test. The growth inhibition zones as recorded in the results were the average of the replications.

In-vivo Phytotoxic Check of the Active Antibiotics-Experimental Design

A hydroponic system for phytotoxicity testing was employed. Incorporation of organic matter into the system was kept to a minimum by washing the roots of the plants to be used. This was necessary to decrease the chance for non-specific binding of the antibiotic while in suspension and thus keep the antibiotic levels as high as possible for entry into the plant. Beakers with a capacity of 250 ml. were used as receptacles for the antibiotic suspensions. A mixture of the compound to be tested was suspended in 50 ml. of water. Some compounds required ethanol for solubilization and in these cases it was always used at a rate of less than 3.0% to avoid possible plant side effects. The potato plants used were grown in flats containing a 1:1:1 mixture of peat moss, sand and soil. Eyes from the main tuber were removed with a melon baller (eyeballed) and planted. This produced a single stemmed plant. Russet Burbank seed tubers grown by John Schutter, a Manhattan, Montana area grower, were used. This particular seed had a very low natural incidence of blackleg. Plants approaching the 10-15 cm. height were used in the test. Selection criteria included a healthy appearance, a thick stem, and a good root system. After placement of the plants in the beakers containing the antibiotic suspensions, each unit was allowed to sit at room temperature (22-24°C) in a greenhouse for three days.

The units were checked every 12 hours for the appearance of phytotoxicity.

Phytotoxicity was determined by observing plant symptoms. These included wilting, leaf chlorosis, and a lack of growth or general nonhardiness of the plant as compared with the healthy control. Starting with the lowest concentration showing phytotoxicity, each compound was diluted by increments of 200 μg/ml. and the experiment repeated for another three day interval. That dosage of the compound to be used in further testing was the highest concentration demonstrating no observable phytotoxic symptoms after a period of three days.

Results

In-vitro Plate Bioassay

A series of cultures were used for the in-vitro testing sequence, e.g., Eca (SR-8), Ecc (AK-241), and a mix of the two. The mixture was useful in determining any major changes apparent in the antibiotic's activity with both bacterial varieties present. Table 4, with the most active compounds given at the top of the table, shows the results obtained with the plate bioassay. Streptomycin, oxytetracycline, chloramphenicol, Rondomycin, and penicillin G, were the most active compounds currently on the market. G 418 Sulfate and J 1-20, two experimental gentamycin derivatives, were also quite effective. Smaller zones were formed by kasugamycin, trimethoprim, and fluorocytosine. This was an indication that their activity against the E. carotovora varieties was limited, and in fact, only kasugamycin was carried over into the next stage of the in-vivo plant check. The remainder of the compounds showed

Table 4. Zones of bacterial growth inhibition with the in-vitro plate bioassay of individual antibiotics.

Antibiotic	Inhibition zone (mm) Concentration (g/ml.)								
	1000			500			100		
	Eca	Ecc	Mix	Eca	Ecc	Mix	Eca	Ecc	Mix
penicillin G	12	14	15	8	11	12	6	7	7
streptomycin	10	9	9	10	8	8	5	3	5
oxytetracycline	9	9	9	7	7	7	5	5	3
chloramphenicol	5	9	9	5	6	7	-	1	3
J 1-20	11	9	10	11	8	9	7	5	5
Rondomycin	10	8	9	9	6	7	6	5	5
G 418 Sulfate	11	9	9	9	7	7	5	4	4
fluorocytosine	3	2	2	2	1	-	-	-	-
trimethoprim*	5	5	5	-	3	2	-	-	-
kasugamycin	1	1	1	1	-	-	-	-	-
Sch 15445	-	-	-	-	-	-	-	-	-
erythromycin*	2	3	2	-	2	2	-	-	-
TZC	-	-	-	-	-	-	-	-	-
S-2	-	-	-	-	-	-	-	-	-
Tylosin Tartrate	-	-	-	-	-	-	-	-	-
albamycin	-	-	-	-	-	-	-	-	-
bacitracin	-	-	-	-	-	-	-	-	-
D-L Tartaric Acid (500 mM pH-3.0)	-	-	-	-	-	-	-	-	-
naladixic acid	-	-	-	-	-	-	-	-	-
Bayer #1-4	-	-	-	-	-	-	-	-	-

*All zones showed extensive clearing of the bacteria but not total inhibition except at the 1000 μg/ml. concentrations.

Growth inhibition zones are read in millimeters of radius extending from the outer edge of the inhibition to the edge of the disc. Eca and Ecc overlayer was 4 x 10^8 cfu/ml. while the mix of the two equaled 8 x 10^8 cfu/ml. The plates were incubated at 24°C and read 48 hours after overlayer.

little or no activity in the test and thus were dropped from further testing.

Combination studies showed some increase in the activities of the antibiotics as seen in Table 5. In all but three cases, there was a zone of growth inhibition at least as large as that produced by the least active antibiotic of the pair. The three exceptions were the combinations of streptomycin-chloramphenicol, kasugamycin-chloramphenicol, and G 418 Sulfate-chloramphenicol.

In-vivo Phytotoxicity Check

All antibiotics taken up by the plant produced some phytotoxicity, either externally observable, or internally non-observable except as reflected in plant vigor (57). Table 6 lists the results of the tests for phytotoxicity with each antibiotic showing some activity against Eca and Ecc as determined with the in-vitro plate bioassay. It should be noted that these are barely sub-lethal dosages and that in practice, the chemicals should be used at lower concentrations. These figures did set the basis for a workable starting point for further research with specific antibiotics to be used for plant uptake.

Table 5. Zones of bacterial growth inhibition with the in-vitro plate bioassay of antibiotics in combination.

Antibiotic		Eca	Ecc	Mix	Rating**
		Zone of inhibition (mm)			
streptomycin-oxytetracycline		11	10	7	NC
(6-8 mm.)""	-penicillin G	13	14	14	+
	-Rondomycin	13	9	9	+
	-kasugamycin	10	7	9	+
	-chloramphenicol	8	7	7	-
	-G 418 Sulfate	11	10	10	+
	-trimethoprim	10	10	9	+
	-J 1-20	12	10	11	+
oxytetracycline-penicillin G		12	10	10	NC
(7 mm.)""	-Rondomycin	13	10	7	NC
	-kasugamycin	8*	8*	8*	+
	-chloramphenicol	9*	9	6	NC
	-G 418 Sulfate	11	11	8	NC
	-trimethoprim	6	9*	8*	NC
	-J 1-20	12	11	10	+
penicillin G-Rondomycin		13	13	13	+
(11 mm.)""	-kasugamycin	11	12	11	NC
	-chloramphenicol	14*	10	11	NC
	-G 418 Sulfate	13	14	13	+
	-trimethoprim	11	12	12	+
	-J 1-20	13	13	12	+
	-bacitracin	12	11	12	NC
Rondomycin-kasugamycin		10	8	5*	NC
(7 mm.)""	-chloramphenicol	11	8	7	NC
	-G 418 Sulfate	11	10	10	+
	-trimethoprim	9	8*	7*	NC
	-J 1-20	12	10	10	+
kasugamycin-chloramphenicol		5	6	5*	-
(0 mm.)""	-G 418 Sulfate	8	8*"	7*	NC
	-trimethoprim	4	5	3	+
	-J 1-20	10	9	9	NC
chloramphenicol-G 418 Sulfate		7	6	5	-
(6-7 mm.)""	-trimethoprim	5	7	7	NC
	-J 1-20	7	6	7	NC

Table 5. (Continued)

Antibiotic	Zone of inhibition (mm) Eca	Ecc	Mix	Rating**
G 418 Sulfate-trimethoprim (2 mm.)""	10	6"	6"	NC
(7 mm.)"" -J 1-20 (9 mm.)""	10	11	11	+
Control (0 mm.)""	0	0	0	

*Slight zone of bacterial growth on the outer margin of the zone.

"Mutation evident around the disc with the antibiotic present. Growth inhibition zones are read in millimeters of radius. All combinations are mixed at 500 µg/ml. each. Eca and Ecc overlayer was 4 x 10^8 cfu/ml. while the mix of the two equaled 8 x 10^8 cfu/ml. The plates were incubated at 24°C and read 48 hours after overlayer. NC = No change.

**Rating -- the change in the zone of bacterial growth inhibition resulting from the combinatory effect of the antibiotics versus the single antibiotic.

""Individual antibiotic inhibition zone measurements for use in the combination effectiveness comparison.

Table 6. In-vivo phytotoxicity on potato plants treated with specific antibiotics.

Antibiotic	Highest concentration in μg/ml. without observable phytotoxic symptoms
streptomycin	3500
oxytetracycline	2000
chloramphenicol	250-300
penicillin G	2000
G 418 Sulfate	3000
Rondomycin	4500
kasugamycin	300-500
trimethoprim	No effect to 11,000
Bayer #2	1000
Bayer #3	200

Replicated with 5 plant/antibiotic treatment. The experiments were conducted until the non-observable phytotoxic dosage was reached.

Discussion

Antibiotic Selection

All compounds were selected preferentially for known Gram negative activity, proven or suspected systemic uptake in plants, and ready availability. Most major groups of antibiotic compounds were represented. It must be emphasized that this study was mainly concerned with the development of a simple, useable system for a certified seed program. It is important to note that several hundred additional antibiotics are now available, many containing features which may be attractive for use as plant protectants. These in a sense can be considered a resource, but proper program guidelines are a necessity if a survey of the compounds is to lead to utilization. Also, even though many antibiotics demonstrated useable activity in-vitro, but were not systemic, they should not be discarded as having no use. The possibility exists that they might be extremely effective tuber disinfectants or have some other currently unknown application.

Positive antibacterial activity at 1000 μg/ml. or below was considered acceptable for advancement into the next stages of the procedure. Use of higher dosages would be unsafe and impractical since plant health was negatively affected by many compounds. There were similar zones of growth inhibition using Eca, Ecc, and the mixture of the two, thus, it was feasible to utilize the mixed overlayer in the remainder of the experiments. This facilitated ease of handling and unnecessary duplication by using each bacteria alone. A routine test of this nature is extremely important before advancing further into the assay, as is evidenced by the fact that neither albamycin nor naladixic acid showed any activity against Erwinia, even though both have demonstrated effectiveness against Gram negative organisms.

The in-vitro plate bioassay performed quite satisfactorily, however, some precautions must be taken. Many researchers agree that the sensitivity of the test is dependent upon numerous factors including the temperature at which it is performed, assuring consistent organism growth, the amount of agar in the plates as well as the type of agar, which may affect the diffusability of the antibiotic, and the amount of bacteria used for the plate overlayer (12,33,34,35,41,100). Once these factors have been taken into account, the test can be quite consistent.

The combination studies did not really show what was actually happening with the antibiotics. It was impossible to tell whether or not there was synergism or antagonism as there was no dose-response curve generated for each antibiotic individually. To calculate synergy or antagonism, it would be necessary to utilize several points of the dose-response curves of each of the antibiotics while in combinations. This could be accomplished through the use of an isobolographic curve. Also, there are inherent problems in the use of the plate bioassay system for combination studies. One of the most crucial effects was that the antibiotics were on a diffusion concentration gradient and failed to diffuse past the specific point in the plate as dictated by the gradient (33). This meant that synergism or antagonism could be impossible to detect, even if it was present. In addition to this, the drugs might form complexes with each other and could result in not necessarily a gain or loss of activity, but a possible loss of diffusability into the agar.

One positive point observed on the plates used for the combination studies was the clarity of the growth inhibition zones in the majority of the cases. There were usually no outer zones of bacteria present, meaning that the antibiotics in combination tended to inhibit both Eca and Ecc equally. In the case of G 418 Sulfate when combined with antibiotics of lesser activity, there was an extremely high rate of bacterial (Ecc) mutation around the discs. See Figures 1 and 2. This might help explain some observations noted later in Chapter 5 when the compound G 418 Sulfate resulted in a faster overall spread of the bacteria in the plant. The possibility of its being a mutagen is high. The combinations, while not demonstrating positive synergy or antagonism, did show zones of growth inhibition which were for the most part at least as large as the least active antibiotic of the pair, thus assuring no real loss of activity.

Figure 1. Eca and Ecc mixed inoculum overlayered on a plate of NA and allowed to stand for 24 hours. The antibiotic disc was placed on the plate 15 minutes after overlayer and the resulting zone of inhibition is apparent.

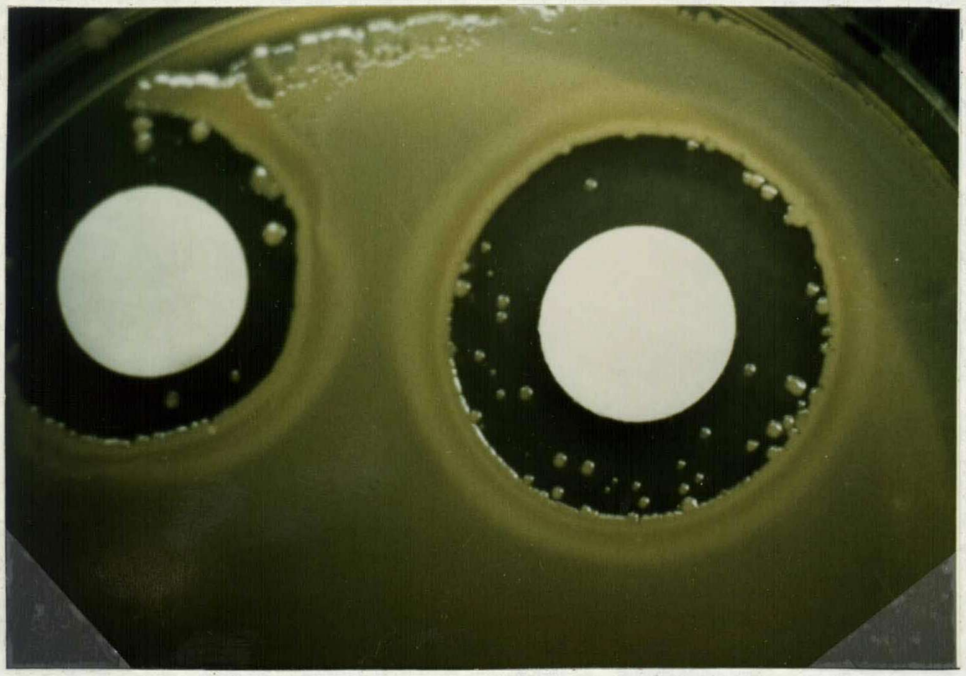

Figure 2. The same plate as in Figure 1 after 72 hours incubation. Antibiotic resistance is evident in the colonies growing in the zone of inhibition.

The antibiotics varied in their ability to ellicit a phytotoxic response in potato. However, the antibiotic could not be useful unless it had activity against the pathogen at or below the highest concentration which did not show visible phytotoxicity. An example of this would be the compound Bayer #3. At 300 µg/ml. it caused plant death, but showed only very limited activity against Erwinia at 1000 µg/ml. Because of the phytotoxicity to the plant, the compound is probably useless as a systemic protectant. When using antibiotics in combinations, there appeared to be higher phytotoxicity in the plants (57). In the case of the streptomycin-oxytetracycline combination, there was increased phytotoxicity at the 1000 µg/ml. level while individually each showed only moderate levels of phytotoxicity at this concentration. However, by reducing the dosage of each slightly, this problem was overcome and the effective protection of the plant was greatly increased. Thus, by using phytotoxicity as a tool for evaluating the antibiotic-plant relationship, the researcher can better decide whether the antibiotic could become an effective systemic agent.

Conclusions

Information on antibiotics to be used in the test procedure was compiled from textbooks and references, research papers, and chemical company manufacturing data. The in-vitro plate bioassay turned out to be a very effective method for assaying antibiotic effectiveness against the blackleg pathogen. It proved to be an accurate, reliable indication of antibiotic activity and was reproducible when replications were performed. Using each antibiotic individually, seven were found which demonstrated any substantial activity against Erwinia. These were penicillin G, streptomycin, oxytetracycline, chloramphenicol, Rondomycin, J 1-20, and G 418 Sulfate. With the drug combinations, it was determined that a liquid assay system providing intimate contact between the bacteria and antibiotics would have been preferential to the use of the in-vitro plate bioassay. All of the antibiotics utilized did show phytotoxic symptoms on potato. Some produced more plant damage than others, but all had the basic symptomology, including, germination and root inhibition, root and shoot stunting, root thickening, wilt, and leaf chlorosis. Dosages of the promising antibiotics were determined which were below the concentrations producing visible phytotoxic plant effects. These were then utilized in tests reported in Chapter 4 for in-vivo plant assays on the activity of the antibiotic in the plant.

CHAPTER 4

ASSAY FOR ANTIBIOTIC SYSTEMICITY

In-vivo Bioassay for Antibiotic Systemicity

The use of a bioassay to determine antibiotic activity in a plant is essential. The antibiotic should be shown to be systemically carried into the plant for the assay to be meaningful. The compound must reach distal areas of the plant, the upper stem and leaf, in high enough concentration to be effective in control of the pathogen. This, of course, varies from one antibiotic to the next, but a plant-plate bioassay should help give an accurate picture of drug movement in the plant.

Materials and Methods

Experimental Design

The hydroponic system, described in Chapter 3, was used and the seed source was the same. Plants were grown from eyeballed seed pieces until they reached the 10-15 cm. stage. Only those with thick, healthy stems and leaves were used. Also, stem cuttings were taken from the plants. With the rooted plants, the seed piece was removed to simulate field conditions. Both rooted plants and stem cuttings were placed in an antibiotic solution as discussed in Chapter 3. Each unit was allowed to sit at room temperature (22-24^{o}C) in a greenhouse for a period of 48 hours. Plants were sampled at intervals of 2, 4, 6, 8, 24, and 48 hours. Six plants were utilized per antibiotic treatment allowing only one replication per time period. Both the upper stem and middle to top-most leaves of the plant were used at each interval for observation. As a different plant from each unit was used at each time interval, some inconsistencies in antibiotic uptake were observed.

Data Collection

The harvested tissue was placed in a press plate for use in a Greenard Laboratory 30 ton press. One half of each tissue sample was squeezed and assayed fresh, while the other half was squeezed, placed in a freezer for 24 hours, and then assayed. An in-vitro plate bioassay as described in Chapter 3 was then performed. Antibiotic sensitivity testing discs were soaked in the plant juice and placed on plates of Mueller Hinton medium overlayered with the blackleg organism. The pathogen mix of Eca and Ecc was overlayered at an inoculum density of 0.1 ml. of 8 x 10^8 cfu/ml. The procedure followed from this point on was the same as described in Chapter 3. Each assay plate also included a control disc soaked with 1000 μg/ml. of the antibiotic being tested. Growth inhibition zone radii were measured in millimeters from the edge of the sensitivity disc to the outer edge of the zone. Measurements were compared with previous zones obtained in Chapter 3 to establish an approximate concentration of antibiotic in the tissue.

Results

The results of these experiments are tabulated in Tables 7 and 8. Table 7 shows data obtained using rooted plants with the fresh plant juice listed first and the frozen plant juice listed in brackets. Table 8 shows data obtained from the stem cuttings with the fresh plant

Table 7. The uptake of antibiotics in-vivo by rooted plant samples as determined by an in-vitro plate bioassay.

Antiobiotic	Dose µg/ml.	Inhibition Zones (mm)											
		Time Intervals for Treatment (hours)											
		2		4		6		8		24		48	
		*L	S	L	S	L	S	L	S	L	S	L	S
streptomycin	2000	**0(0)	2(1)	0(2)	5(4)	2(1)	4(5)	8(7)	6(9)	2(1)	5(8)	9(2)	7(10)
	1000	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(7)	0(0)
	500	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
oxytetracycline	2000	3(3)	2(3)	3(2)	4(3)	3(0)	3(4)	3(0)	5(7)	6(3)	3(5)	5(2)	2(5)
	1000	2(0)	0(0)	2(0)	2(2)	3(0)	3(2)	3(0)	3(2)	5(1)	0(4)	0(2)	1(3)
	500	0(0)	0(0)	2(0)	0(0)	2(0)	0(0)	4(0)	0(0)	5(0)	0(2)	0(0)	0(1)
streptomycin--oxytetracycline	1000	2(2)	2(2)	3(1)	2(1)	6(2)	4(5)	2(0)	1(5)	4(2)	4(3)	3(3)	5(4)
kasugamycin	1000	0(0)	0(0)	0(0)	0(0)	1(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(1)	0(0)
chloramphenicol	1000	6(3)	3(2)	6(6)	6(5)	8(5)	3(8)	5(8)	7(8)	8(10)	9(8)	4(7)	5(5)
Rondomycin	1000	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
penicillin G	1000	11(6)	1(2)	13(12)	5(5)	11(11)	2(4)	9(11)	4(7)	12(12)	3(6)	11(11)	2(2)
streptomycin with 1% glycerol	1000	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	3(0)

*L = Leaf portion   S = Stem portion

**Each unit is given with the fresh squeezing first followed in brackets by the frozen squeezing.

Growth inhibition zones are read in millimeters of radius. Eca--Ecc mix overlayer was 8 x 10^8 cfu/ml.

The plates were kept at a temperature of 24°C and read 48 hours after overlayer.

Table 8. The uptake of antibiotics in-vivo by stem cut plant samples as determined by an in-vitro plate bioassay.

Antiobiotic	Dose µg/ml.	Inhibition Zones (mm)											
		Time Intervals for Treatment (hours)											
		2		4		6		8		24		48	
		*L	S	L	S	L	S	L	S	L	S	L	S
streptomycin	2000	**2(4)	4(2)	0(1)	4(2)	6(5)	5(6)	5(3)	6(4)	7(8)	2(7)	4(10)	3(8)
	1000	0(0)	1(0)	1(4)	2(4)	0(0)	2(2)	2(0)	4(4)	7(7)	2(7)	2(6)	6(4)
	500	0(0)	0(0)	0(0)	0(4)	0(0)	1(3)	0(0)	2(0)	5(0)	5(4)	0(0)	4(0)
oxytetracycline	2000	3(2)	2(3)	3(0)	5(1)	6(3)	2(4)	0(0)	2(4)	7(3)	0(2)	3(5)	0(0)
	1000	1(0)	1(1)	5(3)	3(2)	4(2)	1(4)	6(3)	2(2)	5(4)	1(5)	0(0)	0(1)
	500	0(0)	0(0)	2(0)	1(1)	0(0)	0(2)	1(0)	2(0)	5(0)	1(3)	0(0)	0(1)
streptomycin--oxytetracycline	1000	0(0)	0(3)	0(4)	3(4)	7(4)	4(6)	1(1)	1(4)	6(6)	7(7)	7(2)	6(7)
kasugamycin	1000	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
chloramphenicol	1000	0(0)	2(3)	0(7)	5(6)	6(8)	4(8)	6(8)	3(8)	8(9)	9(8)	0(7)	0(5)
Rondomycin	1000	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(0)	0(0)	0(0)	0(0)	0(0)
penicillin G	1000	7(9)	5(5)	11(11)	4(9)	9(10)	6(7)	9(15)	10(9)	9(7)	8(9)	11(11)	6(8)
streptomycin with 1% glycerol	1000	0(0)	0(2)	0(0)	0(3)	2(0)	3(4)	3(0)	6(9)	2(0)	6(7)	2(7)	2(8)

*L = Leaf portion S = Stem portion

**Each unit is given with the fresh squeezing first followed in brackets by the frozen squeezing.

Growth inhibition zones are read in millimeters of radius. Eca--Ecc mix overlayer was 8 x 10^8 cfu/ml. The plates were kept at a temperature of 24°C and read 48 hours after overlayer.

juice readings also listed first and the frozen plant juice listed in brackets. Differences are evident between the antibiotics at the outset. Streptomycin, as reported by Ark (10), did take time to saturate the available root sites and thus shows a low initial reading but increases after 8 hours. Also, streptomycin at 1000 μg/ml. with 1% glycerol added showed similar uptake patterns as streptomycin at 1000 μg/ml. without the glycerol even though glycerol was reported to improve uptake (64). Oxytetracycline, chloramphenicol, and penicillin G showed early uptake into the plants well within the first 4 hours, with penicillin G having entered the plant in the highest concentration relative to the starting dosage. Freezing versus utilizing the tissue fresh did not seem to demonstrate any major differences. It should be noted that several of the penicillin checks showed some bacterial growth around the disc indicative of possible resistance to the antibiotic. Also, neither Rondomycin nor kasugamycin showed any activity during this phase of the testing and were dropped from further experimentation.

Discussion

Based upon the results obtained, it is evident that the stem cut stocks showed a faster overall uptake of the antibiotics than did the rooted stocks. As stem cuttings are not under the influence of a root system, this is to be expected. It is important to remember, however, that any compromise of the water transport system can dramatically affect antibiotic uptake into the plant and thus limit its potential for entry and protection. Most antibiotics are taken into the plant through the transport system in a passive manner and move through the xylem throughout the plant. Streptomycin is the exception, being able to utilize the active transport system for uptake (57). The results indicate that these ideas are correct as the levels of the antibiotics tended to increase in the upper regions of the plants as time went on, or at the least remain the same.

In-vivo uptake of antibiotics is dependent upon many factors in both the plant and the assay system. The stage of plant growth is critical for antibiotic uptake. Root growth must have proceeded far enough that the seedpiece is no longer necessary as it may act as a reservoir for blackleg bacteria and consequent reinfection after treatment (114). The first stage of plant growth (emergence to tuber initiation) is also the period of fastest water uptake and would be the natural period in growth for rapid antibiotic entry and delivery to all parts of the plant (101). Young plants are easily infected (71) and treatment at this stage can help assure the plant a blackleg-free status as it grows into the more resistant maturing stages.

Root barriers pose the next problem for the assay system. Many chemicals are not absorbed through the root system and thus, other delivery methods must be developed. The first thought was the use of stem cuttings in place of the rooted plants as a means for avoiding the barrier in the roots. However, the main drawback to the use of the stem cuttings is the reduction or inhibition of root formation that occurs after treatment as many antibiotics can be very pronounced in these respects (23).

The assay system itself posed the most pertinent problems. The system was developed so that inhibition of antibiotic uptake attributed to extraneous factors, such as soil binding and excessive heat or light breakdown, was minimized (74). A hydroponic system was accepted, as it gave the fewest problems with either the plants or the antibiotics. Plants washed free of debris before placement in the system were effectively free of antibiotic binding by anything other than the roots themselves.

Plant parts were checked at the locations of naturally occurring infections. The roots, being saturated with the antibiotic, were not assayed. Leaves and stems were squeezed as described by Crowdey and Pramer (38). They reported the press able to recover 53-74% of the sap from the plant part squeezed. This, along with the fact that liquid uptake by the sensitivity testing discs was uniform, showed that most of the inconsistencies which were evident were the result of plant to plant variation. Factors such as leaf size, stem thickness, and the amount of absorbing root surface all make a difference in antibiotic uptake and the amount present in the plant tissue. Time interval sampling was employed, painting a picture of antibiotic uptake rates and partial longevity in both the rooted and the stem cut plants. This allowed for the determination of an optimum treatment time of the plant in the antibiotic. It might be reiterated at this point, however, that the main objective of this phase of the research was not to precisely identify the amount of antibiotic being taken up by the plant, but to establish a definite pattern of plant uptake.

One final caution with the in-vivo plant assay should be addressed. There are definite limitations associated with sap removal from the plant. As 100% of the plant juice cannot be removed and there will be some binding of the antibiotics to the plant parts (57), there is the possibility of obtaining a negative reading for the chemical in the plant, when there may be low levels present. An acceptance of these inherent problems may provide explanations as to why some antibiotics which show poorly in the in-vivo assay do show some control in the challenge with Erwinia as reported in Chapter 5.

Conclusion

It appeared that all of the antibiotics used except Rondomycin and kasugamycin did show some uptake into the plant system. Streptomycin had a definite lag period before entering the plant while the other antibiotics showed more rapid entry and uptake into the system, usually within the first four hours. Both rooted and stem cut plants were utilized. Rooted plants tend to be slower in their uptake of the antibiotics, most likely due to the root barrier. This was especially true in the case of streptomycin. In all cases, the stem cutting's uptake of water and chemical was faster than its rooted counterpart. Freezing of the tissue versus using fresh tissue in the plant sap extraction procedure showed very minimal differences. Some variability was noted among individual plants or time periods and treatments, but these may be accounted for by the differences in the amount of plant tissue used as well as the inherent difficulties in the squeezing technique. More than one plant per time interval/treatment should be used to alleviate some of these problems and give a clearer picture of antibiotic uptake patterns.

CHAPTER 5

EXPERIMENTS IN CHEMOTHERAPY

The Use of Chemotherapeutic Agents to Control Erwinia in Potato and Follow-up Assay for Bacterial Presence

The final phase of testing an antibiotic's effectiveness in pathogen control is the use of infected plants. A three-phase approach was utilized. The first phase was direct placement of the plant in the antibiotic suspension for 24 hours (dosage as determined by Chapter 4 results) and the subsequent challenge of the plant with an Erwinia inoculation. Plant placement in the antibiotic suspension and immediate challenge with the bacterial inoculation was the second phase. The third phase consisted of an inoculation with Erwinia, waiting for visible symptoms to appear, and then, placement in the antiobiotic suspension to demonstrate a lessening of symptoms or remission.

To implement a program of chemotherapy into a certified potato grower's operation, it must be cost effective and show little or no pathogen survivability in the plant. Levels of disease which have dropped to insignificance may be acceptable.

Materials and Methods

Plant Selection and Pathogen Storage

Potato plants produced from eyeballed seed pieces were used at the 10-15 cm. stage of growth. Only those with thick, healthy stems and leaves and well-established root systems were acceptable. Both stem cutting plants and rooted plants were used in the first test, while only rooted plants were used in the second test. The Erwinia organism utilized for inoculation was first grown on CVP to check for pectin hydrolysis and then transferred to Nutrient Agar for growth and subsequent suspension in sterile water for injection purposes. This step was critical in that a fresh preparation of inoculum is needed to assure a good infection in the plant.

For short term studies with the organism, an effective way to store the Erwinia inoculum consists of placing a suspension of at least 4 x 10^8 cfu/ml. ($OD_{640} = 0.1$) in sterile water stored in the refrigerator (5°C) to be reisolated each time use is necessary. The inoculum can be maintained in this fashion for a period of 3 to 5 months. Longer term storage involves placement of the organism in sterile Nutrient Broth, subsequent growth, and then the addition of 10% sterile glycerol to the medium and placement in the freezer (-5°C). Our strains of Erwinia carotovora survived in this frozen state for at least one year.

Antibiotic Preparation and Plant Inoculation

The antibiotic suspensions were prepared in the same manner as given in Chapter 3. The plants were kept in 250 ml. beakers in either water or the antibiotic suspension except in the case of phase 3 where an entire flat of potatoes was placed in the humidity chamber (100% RH) during the inoculation period. This humidity chamber was in a greenhouse with average temperatures at 24°C for the Eca inoculation and 30°C for the Ecc inoculation. Both Eca and Ecc were used, even though the antibiotics demonstrated similar activities toward both. Inoculum densities between 4 x 10^8 to 8 x 10^8 cfu/ml. were used throughout the testing as they were the most reliable for expression of symptoms. A 10 ml. Leur Lock syringe with a 22 gauge needle was used for the inoculation procedures. The bacterial suspension was drawn into a syringe and injected into the stem of the plant at a marked spot on the surface, above the lowest leaves but well below the upper leaves. A hanging drop was suspended from the needle and placed on the surface of the inoculation site. The needle was pushed through the stem at this point and the drop was drawn into the stem. On the other side of the stem, another hanging drop was expressed and the needle was drawn back through the stem and removed. At this point, the plant was placed either in a mist chamber at 100% RH. or a plastic bag was placed over the plant and beaker to maintain a high humidity. Temperature was a critical factor at this stage. Eca must be kept in the range of 18-24^{o}C and can only go as low as 16^{o}C, while Ecc does well at room temperature (24^{o}C) with an optimum of 30^{o}C. Unless both the temperature and humidity are kept well with these tolerance levels, infection will not occur.

Experimental Design

Each phase was set up in a similar manner, but with enough minor differences to make a separate discussion of each appropriate.

Phase 1) Potato plants were placed in antibiotic suspensions for a period of 24 hours as determined in Chapter 4 and then inoculated as stated above. Five plants were placed in each beaker with a total of ten plants used in the rooted form and five plants stem cut for a total of fifteen plants/treatment. Prior to inoculation, each beaker was filled with clean water and the plant roots washed. After inoculation, the beakers were placed in the mist chamber at 100% RH as stated earlier. Temperatures and greenhouse conditions were as described earlier for

Eca and Ecc. An untreated, uninoculated control for both the stem cuttings and the rooted plants was included as well as an untreated, uninoculated control for both.

Phase 2) Potato plants were placed in antibiotic suspensions for 24 hours as described in phase 1, but at the same time as they were inoculated. The same number of plants per treatment were used and all beakers were placed in similar conditions as in phase 1. Controls were also included.

Phase 3) Potato plants were inoculated individually and the entire flat of potatoes placed under conducive environmental conditions until the first sign of visible infection (Figure 3). The infected plants were then removed and placed in the antibiotic suspensions for a period of 24 hours outside of the humidity chamber, at the specific temperatures as described earlier for Eca and Ecc. They were then transferred to tap water and placed back in the humidity chamber for another 48 hours. Proper controls as given above were utilized throughout this procedure. Also, a similar number of plants per treatment were utilized.

Data Collection

A series of two experiments for each phase was performed with Ecc. The first experiment included rooted plants and stem cuttings, while the second experiment only included rooted plants. Antibiotics were changed between experiments as information was accumulated showing antibiotic ineffectiveness. In the case of Eca, only phase 3 was performed even though information from Ecc demonstrated that phase 1 had the best initial control with this type of experiment. Because inoculation and subsequent symptom development were extremely inconsistent with the Eca

Figure 3. Typical inoculated blackleg infection showing the top rot symptoms associated with the stem inoculation technique.

organism, only phase 3, where symptoms could be previewed before using the plants was utilized. It was expected that Eca and Ecc would show similar results in any case as determined by results given in Chapters 3 and 4. Again, two experiments were done, with no stem cuttings included. The reasoning behind these decisions is in the discussion section.

Data were collected on the basis of a visible infection in the cases of phases 1 and 2 or a remission or maintenance of the symptoms in phase 3. The plants were scored on the basis of whether symptoms could still be seen or had remissed and the overall appearance of the plants. Symptoms included a whitish ooze coming from the interior of the infection site, a blackening of the stem around the inoculation site, and a definite wilting of the plant as the infection became systemic. All plants were sliced with a clean, sterile razor blade down the center of the infection site to observe systemic conditions and, any which appeared negative, were transferred to CVP medium for testing. The plates were held anaerobically and a positive reaction was considered as evidence of viable organisms remaining. Two approaches were followed to determine a viable infection. The negative inoculation site was cut out with a sterile knife and placed in a mortar and pestle to be ground up in sterile water releasing any bacteria which may have been present. This mixture was treated as described above. If the inoculation site appeared to be totally clean and hardened, an enrichment technique was utilized. In this case, the injection site was removed and treated as above, but was then placed in Nutrient Broth and allowed to incubate for 48-72 hours at 24^{o}C. At the end of the time period, the NB culture was transferred to a CVP plate, incubated anaerobically for 72 hours, and read along with the others. Once again, pectin hydrolysis was taken to be a positive reaction. Results were recorded and each antibiotic treatment was then given a rating of from 0 to 3. This rating was the culmination of all of the testing performed on a given antibiotic. 0 indicated no control of the blackleg organism while 3 showed optimum control and no evidence of living bacteria still remaining.

Results

Tables 9 through 12 give the data comparing disease reactions under each condition as described in the materials and methods. These tables include data from both stem cuttings and rooted plants. Each table will be explained separately and, on the basis of these results, an overall picture of the critical factors necessary for an antibiotic system will be developed.

When there was a simultaneous application of the antibiotic and the inoculum (Table 9), there was variability evident in the data. A delayed entry time as well as a lack of chemical entry in a high enough concentration to be effective with the lower dilutions of streptomycin allowed a definite increase in disease symptoms. Oxytetracycline at both dosages ellicited symptom development in only 3 of 10 plants showing some definitive control of blackleg as compared with the 9 out of 10 plants showing symptoms in the check. Some phytotoxicity was evident when streptomycin and oxytetracycline were mixed, but overall disease control was excellent. In the second experiment of the series, the

Table 9. Effect of simultaneous antibiotic treatment and inoculation with Erwinia carotovora var. carotovora on blackleg symptom development in potatoes.

Antibiotic Dose (μg/ml.)		Number of Plants with Symptoms Per Total Number of Plants Used			
Experiment One		Rooted	CVP Reaction	Stem Cuttings	CVP Reaction
streptomycin	1000	4/10	-	0/5	-
	500	5/10	-	2/5	-
oxytetracycline	1000	2/10	-	0/5	-
	500	1/10	-	0/5	-
streptomycin-oxytetracycline	1000 ea.	0/10	-	0/5	-
kasugamycin	1000	0/10*	-	0/5*	-
chloramphenicol	1000	0/10	-	0/5	-
Control (no treatment)		9/10	+	5/5	ND**
Experiment Two					
streptomycin	1000	9/10	-		
	500	10/10	ND		
oxytetracycline	1000	4/10	-		
	500	9/10	-		
streptomycin-oxytetracycline	1000 ea.	3/10	-		
	500 ea.	4/10	-		
chloramphenicol	1000	4/10	-		
	500	3/10	-		
penicillin G	1000	10/10	ND		
	500	10/10	ND		
Control (no treatment)		15/15	ND		
Control (no inject)		0/5	-		

*Some phytotoxicity evident in the plants, especially the leaves.
**ND = Not determined as there were no negative readings obtained.
All samples were left in the mist chamber after treatment for a period of 51 hours to allow for symptom development.

Table 10. Effect of 24 hour antibiotic treatment and subsequent inoculation with Erwinia carotovora var. carotovora on blackleg symptom development in potatoes.

Antiobiotic Dose (μg/ml.)		Number of Plants with Symptoms Per Total Number of Plants Used			
Experiment One		Rooted	CVP Reaction	Stem Cuttings	CVP Reaction
streptomycin	1000	1/10	-	0/5	-
	500	0/10	-	0/5	-
oxytetracycline	1000	2/10	-	0/5	-
	500	0/10	-	0/5	-
streptomycin-oxytetracycline	1000 ea.	0/10*	-	0/5*	-
kasugamycin	1000	0/10*	-	0/5*	-
chloramphenicol	1000	0/10*	-	0/5*	-
Control (no treatment)		7/10	+	4/5	+
Experiment Two					
streptomycin	1000	1/10	-		
	500	9/10	-		
oxytetracycline	1000	0/10*	-		
	500	1/10	-		
streptomycin-oxytetracycline	1000 ea.	0/10*	-		
	500 ea.	0/10*	-		
chloramphenicol	1000	0/10*	-		
	500	0/10*	-		
penicillin G	1000	0/10	-		
	500	0/10	-		
Control (no treatment)		15/15	ND**		
Control (no inject)		0/5	-		

*Some phytotoxicity evident in the plants, especially the leaves. Some wilting was also evident.

**ND = Not determined as there were no negative readings obtained.

All samples were left in the mist chamber after treatment for a period of 48 hours to allow for symptom development.

Table 11. Effect of antibiotic treatment on blackleg symptom development in potato plants previously inoculated with Erwinia carotovora var. atroseptica until visible symptoms were evident.

Antibiotic Dose (μg/ml.)		Number of Plants with Symptoms Per Total Number of Plants Used	
		Rooted	CVP Reaction
Experiment One			
streptomycin	1000	1/10	-
	500	1/10	-
oxytetracycline	1000	6/10*	-
	500	0/10*	-
chloramphenicol	500	0/10	-
	250	0/10	-
penicillin G	500	0/10	-
	250	2/10	-
streptomycin-penicillin G	500 ea.	0/10	-
chloramphenicol-penicillin G	250 ea.	1/10	-
streptomycin-oxytetracycline	500 ea.	0/10*	-
Control (no treatment)		5/10	2 (-) & 3 (+)
Experiment Two			
streptomycin	1000	1/10	-
	500	1/10	-
oxytetracycline	1000	7/10*	-
	500	0/10*	-
chloramphenicol	500	2/10	-
	250	1/10	-
penicillin G	500	0/10	-
	250	2/10	-
streptomycin-penicillin G	500 ea.	3/10	-
chloramphenicol-penicillin G	250 ea.	4/10	-
streptomycin-oxytetracycline	500 ea.	0/10*	-
Control (no treatment)		6/10	1 (-) & 3 (+)

*Some phytotoxicity evident in the plants, especially in the leaves. All samples were left in the mist chamber after treatment for a period of 48-60 hours to allow for symptom remission or disappearance.

Table 12. Effect of antibiotic treatment on blackleg symptom development in potato plants previously inoculated with Erwinia carotovora var. carotovora until visible symptoms were evident.

Antibiotic Dose (μg/ml.)		Number of Plants with Symptoms Per Total Number of Plants Used			
Experiment One		Rooted	CVP Reaction	Stem Cuttings	CVP Reaction
streptomycin	3500	0/10	-	1/5	-
	1000	0/10	-	1/5	-
	500	0/10	-	0/5	-
oxytetracycline	3500	1/10	-	1/5	-
	1000	0/10	-	3/5	-
	500	0/10	-	1/5	-
streptomycin-oxytetracycline	1000 ea.	1/10	-	2/5	-
kasugamycin	1000	0/10	-	1/5	-
chloramphenicol	1000	1/10	-	1/5	-
G 418 Sulfate	1000	10/10	ND**	5/5	ND
Control (no treatment)		10/10	ND	3/5	+
Experiment Two					
streptomycin	1000	2/10	-		
	500	2/10	-		
oxytetracycline	1000	3/10*	-		
	500	2/10	-		
streptomycin-oxytetracycline	1000 ea.	3/10*	-		
	500 ea.	2/10	-		
chloramphenicol	1000	3/10*	-		
	500	1/10*	-		
penicillin G	1000	3/10	-		
	500	4/10	-		
Control (no treatment)		7/15	2 (-) & 6 (+)		
Control (no inject)		0/10	-		

*Some phytotoxicity evident in the plants, especially the leaves.
**ND = Not determined as there were no negative readings obtained.
All samples were left in the mist chamber after treatment for a period of 48-60 hours to allow for symptom remission or disappearance.

infection rate was much higher. Explanations for this will be given in the discussion.

The stem cuttings of the first experiment showed good control of the pathogen and thus probably demonstrated good antibiotic uptake. Only plants treated with the low concentration of streptomycin (500 g/ml.) showed any symptom development.

Table 10 lists the results of the antibiotic dip for 24 hours and then subsequent challenge with Erwinia (phase 1). Extremely consistent results were obtained in this procedure with both stem cuttings and rooted plants. If the antibiotic was able to penetrate into the stem, then protection from infection was evident. Some phytotoxicity was evident with both chloramphenicol and the oxytetracycline-streptomycin mixture, but these symptoms might have been alleviated with lower dosages of each. Streptomycin at 500 μg/ml. demonstrated effective control of the pathogen in the first experiment, but not in the second. This may point to erratic uptake, particularly with the lower dosages.

Results from the antibiotic treatment after visual symptoms have been obtained (phase 3, Table 11) using Eca and rooted plants showed varying results. Control of the pathogen was quite high except in the case of oxytetracycline at 1000 μg/ml. where the plants showed extensive decay in both experiments. It was probable that the plants were unable to provide any effective resistance against the blackleg organism, even with the antibiotic present. This was due to the excessive phytotoxicity evident, probably resulting from a reaction to a high ethanol concentration in the antibiotic suspension.

The poorest overall control of the Ecc pathogen was obtained using the antibiotic treatment after visual symptoms had been ellicited. Table 12 outlines the results. Plants utilized in the first experiment demonstrated reasonable control of the pathogen in the rooted form, but they developed high infection rates in the stem cutting form. All plants in the first experiment were placed in the antibiotic suspension at the first indication of visible symptoms. Each plant was observed on a six hour basis to preview symptom expression before antibiotic treatment. Of all the treatments in the first experiment, the G 418 Sulfate was the one exception to good control. There was actually a spread of the blackleg bacteria to all parts of the plant and there was a 100% infection rate. There was a strong indication that the compound adversely affected the plant's defense system and was probably a mutagen as it is related to the gentamycin compounds which are strong mutagens. In the second experiment, symptoms were allowed to progress to a more advanced state and then the plants were utilized. Higher levels of infection were seen as compared to the results of the first experiment. In all cases, at least one plant of the ten samples showed symptoms still remaining after treatment, and in the case of penicillin G at 500 µg/ml., a total of four plants showed symptoms.

Results obtained with the CVP inoculations from the plants with negative readings are listed in each of the respective tables. In all cases, visible stem symptoms were taken as positive readings and recorded. All other stem readings were considered as visual negatives and were biochemically checked to assure no viable organisms were present. As is evident, all of the follow-up checks proved to be negative with the exception of the controls, which were not treated. Table 13 summarizes the ratings of the antibiotics after examining all of the preceding results throughout the thesis. It is evident that the four systemic compounds, streptomycin, penicillin G, chloramphenicol, and oxytetracycline as well as the streptomycin-oxytetracycline mixture were rated the highest. The remainder of the chemicals were rated quite low because of mutagenic properties, phytotoxicity problems and lack of plant uptake.

Table 13. Summary of the antibiotic ratings (0-3) based upon the antibiotic's overall performance in the control of blackleg of potatoes.

Antibiotic	Rating (0-3 with 0 = lowest and 3 = highest)
penicillin G	3
streptomycin	3 (higher dosage)
oxytetracycline	2'
chloramphenicol	3'
streptomycin-oxytetracycline	3'
streptomycin	1 (lower dosage)
kasugamycin	1'
Rondomycin*	0
G 418 Sulfate	0

*There was no uptake demonstrated, but this particular antibiotic may show some promise as a tuber disinfectant.

'These antibiotics showed phytotoxicity at the dosages used and should be tested at lowered dosages to see if phytotoxicity can be decreased without losing effective pathogen control.

Discussion

Large differences were obtained between experiment one and two with the simultaneous antibiotic application and inoculation as far as symptom development was concerned. These could be explained quite simply. When the jars were placed in the mist chamber for the second experiment, they collected large quantities of water within the beakers and the antibiotics may have been diluted to concentrations below the protective level. As far as the overall system was concerned, it probably was not ideal. Expectation of high water uptake by the plant when surrounded by an atmosphere of 100% relative humidity was unrealistic. The plant was unable to establish a water transpirational pull to amount to anything as there was essentially a non-existent humidity gradient, thus chemical uptake was probably kept to a very basic diffusion gradient. Unless the antibiotic was able to enter the plant rapidly and in very high initial concentrations, as with penicillin G or chloramphenicol, there was little protection against bacterial infection. Also, once the systemic infection was established, actual water transport may have been cut to a minimum, hampering the antibiotic's ability to enter the plant and its corresponding ability for protection.

The data given in Table 10 showed that by far the most effective means of determining antibiotic effectiveness was the 24 hour antibiotic treatment and subsequent inoculation with Erwinia. Use of this method allowed antibiotic entry into the plants at levels which provided very effective control of Ecc. Based upon the results seen with this phase, it was reasonable to expect that if antibiotic uptake occurred with a fully operational water transport system, the blackleg organism could be controlled. Therefore, antibiotic control of the blackleg pathogen seems a very real possibility. It should be mentioned that there was control of the pathogens with antibiotics which showed little or no uptake in-vivo, streptomycin at 500 μg/ml. being a good example. This serves to illustrate limitations present in antibiotic detection in the plants with this system.

Phase 3 or the effect of antibiotic treatment on potato plants after obtaining a visible infection process (as shown in Tables 11 and 12) gave interesting insights into the workings of the plant-antibiotic system. Differences were evident in the results obtained between the first and second experiment and the stem cuttings showed a much higher infection rating than in the other two phases. In the first experiment, the plants were removed and utilized at the first sign of visible infection. At this point the water transport system was still reasonably intact and fairly good antibiotic uptake occurred. In the second experiment, plants were not taken until the visible infection could be clearly seen. The water transport system was compromised and thus pathogen control was not as effective as in the first experiment. In the case of stem cuttings, the water transport system was also involved, but in a different manner. Because of the severe upper leaf wilt, it was evident that little water transport occurred into the stem cuttings and thus antibiotic movement was probably minimal as was pathogen control. If there was any malfunction of the water transport system, varying rates of antibiotic uptake would most likely occur, and protection to the plant would be minimized. In a grower's situation, plants should be selected for treatment which are healthy and vigorous, allowing for good uptake to occur. Nonetheless, even with lowered uptake rates, the systemic movement of the pathogen into the plant was slowed somewhat as compared with the control and did show slightly less disintegration of the surrounding tissue.

The use of enrichment techniques coupled with inoculation of the plant juice to CVP medium provides a suitable system for assaying bacterial populations which may remain in the plant after treatment. As all of the CVP readings were negative, one of two conclusions could be drawn. Either there were no living bacteria present (Erwinia) at the infection site, or they were in such low numbers that detection with this method was not possible. As this test has proven to be quite reliable, it is probably safe to assume that there were insignificant numbers of Erwinia present, if any. This also points to the effectiveness of reading visible symptoms as a means for diagnosing bacterial presence in the plant. By slicing the stem lengthwise above and below the inoculum site, a fairly accurate picture of the infection process was gained. It might be noted that a browning reaction around the inoculation site as well as a walling off process by the plant tissues was the common indication of failure of the bacterium to infect.

Antibiotic ratings provided an overall assessment of the ability to systemically protect a potato plant from blackleg. Antibiotics which were rated high (2-3) were all systemic in action. The other antibiotics which were rated lower demonstrated problems ranging from no uptake at all in the case of Rondomycin, to excessive phytotoxicity with kasugamycin, and no pathogen control with G 418 Sulfate. These types of problems show a definite need for assay programs of this type whenever working with antibiotic-plant systems. Also, the importance of antibiotic combination studies becomes evident, as effective antibiotic dosages can be lowered and reduce phytotoxicity.

Conclusions

Three different challenge experiments were conducted with the selected antibiotics. Of the three, the antibiotic dip for 24 hours with a subsequent challenge of *Erwinia* was the most accurate method for determining antibiotic activity in-vivo. Once the antibiotic entered the plant, control was easy to obtain. Using the simultaneous antibiotic dip and *Erwinia* challenge was somewhat effective, but left one unsure whether or not proper chemical uptake had occurred. Post infection treatment of the plants was the most severe test of the three and also the least accurate method for determination of antibiotic effectiveness. Probably once the water transport system was compromised, compound uptake became minimal and control was marginal. In all cases, excepting G 418 Sulfate, there was a lessening of disease severity and symptom expression after antibiotic treatment. With the use of G 418 Sulfate, there was actually a spread of the pathogen throughout the plant, showing a much more severe reaction than even the infected controls which were untreated. This may be due to the mutative properties of the compound as seen in the in-vitro testing of the antibiotic with Ecc, or, to a possible breakdown in the plant defenses when in contact with the compound. Very good control of the challenge organism was observed in all but the third phase with the stem cuttings. Little difference was observed between the rooted and the stem cut stock, other than the fact that the stem cuttings allowed a more rapid antibiotic uptake. Also, similarities were evident in the reaction of Ecc and Eca to each antibiotic. Overall, the best situation for in-vivo testing of an antibiotic's effectiveness is to first allow for plant uptake and then challenge the plant with the test organism.

In the determination of the presence of viable Erwinia at the injection sites which were symptomless, it was concluded that no living bacteria were found using a biochemical system. Given the proper conditions, including temperature and humidity, the infection, if viable, should manifest itself in a visible manner. Symptoms generally were very clear cut indications of a living Erwinia organism. It was concluded that Erwinia presence as dictated by visible symptoms was as accurate a method of detection as the biochemical CVP method.

Using a rating procedure which took into account all factors in the assay of an antibiotic for systemic action against Erwinia, four antibiotics were found to be quite effective. These were streptomycin, oxytetracycline, penicillin G, chloramphenicol, and a streptomycin-oxytetracycline mixture.

CHAPTER 6

PLANT WATER UPTAKE VERIFICATION

Utilization of a Dye Marker for the Assurance of Proper Water Uptake

A dye marker, or a compound which will show its presence in the plant when proper uptake has occurred, is of importance in the system as a way of making certain that all of the plants take up antibiotics through a viable water transport system.

Materials and Methods

Experimental Design of the Dye Marker System and Data Collection

To determine optimum concentrations of the acid fuchsin dye, a hydroponic system similar to the one performed in the previous chapters was utilized. Eleven concentrations of dye ranging from 0.001% to 1.0% were checked for uptake into the plant and amount of color in the leaves. The dye was mixed with 50 ml. of water in 250 ml. beakers and individual 10-15 cm. potato plants produced from eyeballed seed pieces were placed in the solutions. The plants were observed at 30 minutes, 2, 8, and 24 hours. At the end of the observation period, the plants were removed from the dye, the roots washed, and the plants placed back into beakers filled with clean tap water. They were again observed for a period of 3 days to look for the displacement of the dye residue from the interior of the leaves and stem to the outer margin of the leaves. Each concentration of dye was analyzed for its time to reach the leaves, the amount of color it left in the leaves, and the time it took for the dye residue to be displaced to the outer leaf margins if it occurred. Also important was the appearance of the plant after the dye treatment, i.e., that no phytotoxicity was evident. These experiments were carried out in a greenhouse at temperatures of 24-33°C and at a relative humidity of approximately 50%.

These results were compared with the time it took selected antibiotics to reach adequate levels in the plants. The optimum dye concentration was then selected and mixed with the different antibiotics at the dosage utilized in the testing given in Chapter 5 and a comparison of the dye uptake versus antibiotic uptake was made. Again a hydroponic system was utilized with five plants per treatment. In each treatment, one plant was sampled at each time interval of 4, 8, 12, and 24 hours, with the last plant left for a total period of 72 hours to observe any developing phytotoxic reactions. The in-vitro plate bioassay was used to determine levels of the antibiotic present at the time intervals corresponding to color build-up in the leaves of the plants. Both fresh tissue and frozen tissue from each plant were squeezed similar to the method utilized in Chapter 4.

Results

Results of the dye uptake into potato plants were quite consistent. The plants with the lower dye concentrations of 0.001%, 0.01%, and 0.02% showed no visible uptake evident in the leaves after a 48-hour period. The plants with the dye concentration of 0.04% demonstrated the first visible effects in the leaves after 8 hours. There was never more than a very partial involvement of the leaves as far as color was concerned. The plants with dye ranging from 0.06% to 0.2% and including 0.08% and 0.1% showed color build-up in the leaves after 2 hours. However, all but the 0.2% level showed only partial involvement of the color in the leaves. Partial involvement meant that the leaves were not totally inundated with the dye. The plants with dye concentrations of 0.4%, 0.6% and 1.0% had color evident in the leaves after 30 minutes. All of these concentrations demonstrated a full involvement of the plant tissue, with the dye build-up totally blocking any normal green color in the leaves. When the plants were washed and placed into tap water, all of the dye was displaced to the outer leaf margins in a matter of hours, before the 72-hour time period was up. The exception to this was the two upper dye concentrations which did not show any normal leaf color after the time period was finished. The dye concentration chosen for potato plant uptake was 0.1% w/v. Dye uptake was most consistent at this level without observed damage to the plant. Figures 4 and 5 show the appearance of the plant during dye uptake (Figure 4) after dye displacement from the leaves (Figure 5). Table 14 lists the data obtained from the in-vitro plate bioassay of the plants taken from the dye-anti-biotic mixture experiment. Both leaves and stems were shown to have enough of the antibiotic present to inhibit the Erwinia pathogen after 4 hours of treatment. Dye uptake was also complete after 4 hours and a definite red color could be observed. There appeared to be little difference between zones obtained with the fresh squeezed tissue and the frozen, then squeezed tissue.