Annual Report
1992-1993
Innovative Strategies for the Detection and Control of Bacterial Ring Rot

Submitted by:

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Overview of Project

Bacterial ring rot is an economically significant disease affecting seed and table stock production of potatoes in Colorado. It is widely accepted that economic losses due to bacterial ring rot would be much greater if it were not for the success of current certification programs aimed at production and commercial distribution of disease-free seed. Unfortunately, the availability and use of disease-free seed has not eradicated the pathogen, Clavibacter sepedonicum, which is often present in the absence of disease symptoms. There are no chemical control measures for limiting the pathogen in seed used in table stock production, or for preventing certified seed from becoming contaminated by any of several possible sources. Chemical disinfestation of cutting equipment and storage facilities helps to limit spread, but is inadequate to prevent recontamination of seed by bacteria carried in irrigation water or on weeds. The primary objective of my research is the development of innovative strategies for the detection and control of bacterial ring rot.

This is an ongoing project. In 1990, the SLV Research Center Committee allotted funds for the development of a biological control system for C. sepedonicum. In 1991 and 1992, the Committee allotted funds for a continuation of an expanded program aimed at the ultimate goal of managing bacterial ring rot. The following objectives, which reflect my overall bacterial ring rot program, were stated in my 1991 and 1992 proposal.

1) Continue work on the biological control of bacterial ring rot.
2) Evaluate immunity and resistance of Solanum spp. in a potato bioassay.
3) Compare methods for detecting and quantifying the ring rot bacterium.

A progress report for each objective is attached. The introduction and methods are in part the same as those of the 1991-1992 annual report.

Objective 1: Continue work on the biological control of bacterial ring rot.

Introduction

This project was initiated in 1990 and continued in 1991 and 1992. Funds provided by the SLV Research Center Committee were matched in 1991-1992 by a grant awarded from the Colorado Advanced Technology Institute/Colorado Institute for Research in Biotechnology. To be eligible for this award, an awardee had to have prior funding from an industry in Colorado. The overall goal of the project is the development of biocontrol agents that can be applied to seed prior to planting to limit the spread of bacterial ring rot in commercial production and to prevent the recontamination of generation seed.

Biocontrol, utilizing introduced or indigenous microorganisms to suppress disease, is receiving increased attention due to concern over the use of pesticides in agriculture, and because it may provide control for plant diseases that cannot be managed by traditional strategies. Biocontrol of bacterial ringrot has not been attempted previously, but four observations suggest it's feasibility. (1) The vascular system of potato is an ideal niche for many microorganisms. In 1974, De Boer reported the presence of large numbers of nonpathogenic bacteria in the vascular system of healthy potato plants. The ecological significance of these "endophytic" bacteria, some of which are antigenically related to the pathogen, remains unknown. (2) Some of the bacteria present in potato inhibit the growth of C. sepedonicum in culture. Possibly, certain endophytic bacteria are also capable of reducing growth of C. sepedonicum in plants. (3) Ecology studies with C. sepedonicum have enabled identification of specific windows of time in which biological control agents are most likely to be effective. Certified seed potatoes begin as sterile plants, micropropagated through stem cuttings grown in tissue culture. These plantlets are transplanted and grown in greenhouses to produce nuclear seed, and certified for the absence of specific potato pathogens. Subsequent generations derived from nuclear seed are grown under field conditions by certified seed growers. Thus, windows of application occur during production of nuclear seed and during production of subsequent generations. (4) Biocontrol is feasible because biocontrol agents can be applied as root or seed dips prior to planting. Seed piece inoculations have been used successfully to apply bacterial antagonists to control potato soft rot diseases.

The potential of endophytes as biocontrol agents or as biological delivery systems of

agriculturally important chemicals is largely unexplored. Recent studies show that endophytic bacteria are present in cotton, pear, and corn. Crop Genetics International has developed a genetically engineered endophytic bacterium for control of corn ear worm. The product, called INCIDE™, is a strain of C. xyli that multiplies in the vascular tissue of corn and expresses the endotoxin gene from Bacillus thuringiensis. C. xyli and C. sepedonicum are closely related members of the same group of plant-associated Gram-positive coryneform bacteria. The strategy I propose for developing biocontrol agents for bacterial ring rot is similar to the one which resulted in INCIDE™. The differences are that the crop is potato and the immediate target pest is a bacterial disease. After specific strains of endophytic bacteria are shown to multiply to significant numbers in potato, genetic engineering may be used to develop strains for specific applications. Knowledge of mechanisms underlying biocontrol would be especially helpful in defining characters that could be targeted for future agricultural biotechnology research.

Historically, symptom expression of bacterial ring rot in plants grown under greenhouse conditions has been very difficult to reproduce. Soon after starting my studies in 1990, a bioassay was developed to monitor bacterial ring rot in potato. Tissue-cultured potato plantlets serve as a source of sterile, pathogen free starting materials for the assay. Plantlet roots of the susceptible potato variety Sangre are dipped into a suspension of a biocontrol agent, dried briefly, and dipped into a cell suspension of the pathogen. Plants are transplanted and placed in a greenhouse. Bacterial ring rot symptoms appear routinely after 27 days in plants inoculated with the pathogen only. Disease is quantified by the number of symptomatic plants, numbers of days to symptom expression, and proportion of leaves with symptoms.

In 1990, I initiated a program to isolate and identify potential biocontrol activity among endophytic bacteria present in potatoes grown in Colorado. A total of 736 bacteria from surface-sterilized stem sections of potato were isolated and stored during 1990. Samples were collected from 11 potato cultivars throughout the growing season. Bacteria were isolated onto three different media to optimize selection of diverse bacterial species. The number of bacteria present in stems varied between zero and 10 million bacterial cells per gram of tissue. Bacteria growing in potato stems varied with respect to colony morphology, growth rate, and presence on each of the three media. Thus, a wide variety of bacterial types are included in the endophyte collection.

In 1991-1992, the focus of the research was to screen endophytes for biological control activity in potato plants, and to evaluate in vitro antibiosis of C. sepedonicum by endophytes. In 1992-93 those endophytes that looked promising in the first screening were rescreened in laboratory and greenhouse tests and a subset evaluated in field trials at the San Luis Valley Research Center and at the Bay Farm in Fort Collins.

Materials and Methods

Bacterial strains.

Strains of C. sepedonicum and endophytic bacteria were maintained as glycerol stocks at -80C. Bacteria were those isolated in 1990, as described in the 1990 Progress Report. All bacteria were recovered from frozen glycerol stocks by extracting a small chunk of the culture onto an appropriate medium and incubating for 2 days (endophytes) or 5-7 days (C. sepedonicum) at 26°C. A spontaneous rifampicin-resistant mutant (CIC31) of a strain of C. sepedonicum isolate from a potato grown in Colorado was used in most experiments.

Biocontrol activity in potato.

Potato plants of cv. Sangre were micropropagated according to standard procedures. Nodal stem cuttings were grown in MS-sucrose agar for 6-8 weeks in a growth chamber with 16 hr light at 24°C and 8 hr dark at 18°C. Prior to use in the biocontrol assay, plantlets were hardened off by placing them in the cold (40°F) for 2-3 days.

On the day of inoculation, plantlets were removed from the culture jar, and rinsed briefly in sterile distilled water to remove any adhering agar. Plantlets were soaked for 20 minutes in a cell suspension of an endophyte adjusted to an $O.D._{640}$ of 0.1-0.2, (about 10_8 cfu/ml) dried for 10 minutes, and then soaked in a cell suspension of CIC31 adjusted to an $O.D._{640}$ of 0.1-0.2 (about 5×10^7. Four plantlets per treatment were planted individually in 10.2 cm pots containing TerriLite Metro Mix 352. Pots were placed in a mist chamber at about 60°F for 2-3 days in a greenhouse, and then transferred to greenhouse benches. Plants were maintained for 60 days. Number of days to symptom onset, and disease severity and incidence were recorded.

In vitro antibiosis.

Endophytes were suspended in phosphate buffer (20 mM, pH 7.2) to an optical density (O.D.640) of 0.1, and 5 μl of the cell suspension spotted onto two plates each of tryptic soy agar (TSA), yeast extract-glucose medium (YGM), and King's B medium (KMB). Cells were grown at 26°C for 48, killed with chloroform vapors, and covered with 5 ml of molten soft agar (0.7%) of TSA, YGM, or KMB that was inoculated with 100 μl of a cell suspension ($O.D._{640}$ 0.2) of C. sepedonicum strain CIC31. After 5-7 days at 26°C, the presence of clear zones in the lawn of C. sepedonicum surrounding colonies of endophytes was recorded.

Preparation of pathogen inoculum.

CIC31 was grown on TSA agar for 6-9 days at 26C. Cells from several TSA plates were removed with a sterile glass rod and suspended in one quarter strength Ringer's solution. Inoculum was adjusted to an optical density ($O.D._{640}$) of 0.075 which contained about 4 X 10^8 colony forming units (cfu)/ml. Inoculum was used within one hour of preparation.

Endophyte inoculum preparation.

Methods for obtaining cell suspensions of each endophyte were the same as those described above for CIC31, except that the suspensions were adjusted to an O.D.640 of 0.1 (about 1 x 10^8 cfu/ ml).

Field trials.

Tubers of cv. Sangre, obtained as Colorado certified seed (G2), were removed from cold storage 7-10 days prior to the inoculation date to promote bud formation. Tubers were inoculated by vacuum infiltration or by cutting with a contaminated cutting knife. For vacuum infiltration, seeds were sliced longitudinally with a sterile knife, immersed in a 6 liter cell suspension of CIC31 in a vacuum chamber, and a 15 lb vacuum applied for 10 minutes. For knife inoculation, tubers were inoculated by cutting the tuber with a knife that had been dipped in a suspension of CIC31 made in Ringer's solution containing 0.3% Bacto agar and dried overnight on a greenhouse bench. Control plants (inoculated with Ringer's solution only) were prepared and stored before CMS suspensions were made and used to inoculate seed pieces to avoid cross contamination. After treatment with CIC31, seed pieces were soaked for 30 minutes in a cell suspension of an endophytic bacterium and dried overnight in a greenhouse.

Six endophytic bacteria (CA40, CA58, CA82, CA86, CA90, and CA116) were evaluated in field plots in the San Luis Valley and at the Bay Farm in Fort Collins. The experimental design was a randomized complete block with six replications, two inoculation methods, and eight bacterial treatments, which included the six endophytes and seeds treated with CIC31 alone and seeds treated with buffer only. Ninety-six individual plots of ten feet in length and containing ten seed pieces were planted at each location. Spacing between rows was 30" at the Bay farm and 36" at the SLV field. Every other row was planted to increase the between row spacing. Fertilizer was banded about 2-4 inches below the seed piece prior to planting. At the Bay Farm, fertilizer purchased from PureGro was banded to give a 100, 70, 50, 20, 2 of N, P, K, S, and Zn, respectfully, at 442 lbs/acre (0.31 lbs/ten row plot). At both locations, preemergence herbicides and insecticides were applied as needed. Plots in the SLV were planted on 5/8/92 and harvested 8/27-8/28/92. Plots at the Bay farm were planted on 5/1/92 and harvested 8/21/92.

Number of emerged plants and plant height of three plants/plot were measured at eight, nine and ten weeks after planting. The number of plants with foliar symptoms per plot was recorded every week throughout the growing season. At harvest, external and internal symptoms of bacterial ring rot, and yield were recorded. Data were analyzed statistically with M-Stat.

Serological detection of CIC31.

To detect and quantify the populations of CIC31 in stems grown from inoculated seed, an ELISA assay was used to detect C. sepedonicum. A PathoScreen Kit was purchased Agdia, Inc. and used according to manufacturer's recommendations. Two stems from each plot were harvested, placed in a plastic bag, and stored in a cooler before returning to the lab where a stem section containing the first ten centimeters above and below the soil line were excised and stored at -80C. A one gram section of the soil line region of each stem was macerated in one ml of extraction buffer. Samples were diluted ten-fold and stored at -80C until processing.

Results

Characteristics of endophytes with biocontrol activity.

A large proportion of the 117 (61%) endophytes screened to date delayed bacterial ring rot symptom expression by 3 or more days (Tables 1,2). A second screening of the 30 endophytes that were correlated with 100% control of bacterial ring rot symptoms was completed in 1992. Six of these strains gave 100% control after rescreening. These six strains were tested in the 1992 field study. All six strains produced antimicrobial activity against C. sepedonicum when grown in culture. Some strains inhibited C. sepedonicum only on one of the three media used, while others produced an inhibitory compound(s) in all three media. Table 3 summarizes characteristics of the six endophytic bacteria that had consistent biocontrol activity in greenhouse studies.

Field trials of biocontrol.

In general, incidence of foliar and tuber symptoms of bacterial ring rot were greater in the plots at the Bay farm in Fort Collins as compared to foliar and tuber symptoms in plots at the San Luis Valley Research Center (Figures 1,2,3). Significant differences ($P < 0.05$) external or internal bacterial ring rot symptoms, stand counts, plant height, or plant vigor were not detected at either location. Significant differences due to inoculation method were found at most sample dates at both locations. Disease, measured by yield, plant height at 9 and 10 weeks, foliar symptoms, or external and internal tubers symptoms were significantly greater is seed that was vacuum infiltrated with CIC31, as compared to disease in seed inoculated with a contaminated cutting knife. Significant differences due to strain were found at the Bay Farm plots at 92, 98, and 105 days after planting (Figure 1, Table 4). At 110 days, the difference was very close to being statistically significant ($P < 0.055$). Means separations were analyzed by Tukey's Honestly Significant Difference test, which is very conservative, meaning the chance of having a difference but not reporting it is higher than with other testes. The stringency of the test is especially obvious for the 98 day data. Significant differences were detected by an analysis of variance, but differences in means were not detected by Tukey's test (Table 4 and5).

Serological assays.

Significant differences due to inoculation were detected at the San Luis Valley and the Fort Collins locations. ELISA values of stems collected from tubers that were vacuum infiltrated were higher than ELISA values of stems collected from knife inoculated tubers. Significant

differences due to strain were detected in stems from the San Luis Valley, but not in stems collected from the Bay Farm. Results of the ELISA readings are summarized in Figure 4 and Tables 5 and 6

Discussion

Over 700 endophytic bacteria isolated from potatoes grown in Colorado are maintained in a culture collection at CSU. Preliminary screening of these bacteria as potential biological control agents was initiated in 1991 and was continued in 1992. A high proportion of the strains (26% of total number tested) had a negative affect on symptom expression of bacterial ring rot. Five endophytes actually promoted bacterial ring rot; symptoms appeared 3 days prior to symptoms in control plants inoculated with C. sepedonicum alone. These results were encouraging and suggested that ring rot symptoms may be reduced or aggravated by certain endophytic bacteria.

To test the effectiveness of endophytic bacteria as biological controls of bacterial ring rot under field conditions, we chose two inoculation methods that would simulate the types of inoculum commonly associated with bacterial ring rot infections in potato production. The vacuum infiltration method was used to simulate internal seed contamination, and the contaminated knife cut was used to simulate spread of the pathogen during the seed cutting process. Both inoculations methods were extremely successful, however differences in the number of symptomatic plants was less in the San Luis Valley than in Fort Collins. This difference in symptom expression due to location, has been reported by A. Van Buren and M. Harrison (Master's thesis, A. VanBuren)

Another reason we chose to use tow inoculation methods and two locations was to increase the probability that different levels of disease would be established, and thus enable an evaluation of biocontrol over a range of disease pressure. However, other experimental designs are needed to evaluate the effect of disease pressure on efficacy of biological control.

Several of the 198 strains tested to date produce compound(s) that have antimicrobial activity against C. sepedonicum under laboratory conditions. About 70% of the 30 strains identified as potential biocontrol agents in greenhouse studies also killed C. sepedonicum *in vitro*. It is possible that an antibiotic activity effective against C. sepedonicum is involved in the biocontrol activity of some of these strains. In some cases, however, antibiotic activity may not

play a role in biocontrol.

The biocontrol activity in the field was much less than the biocontrol activity obtained in greenhouse studies. Part of the reason for the relatively poor efficacy in field trials may be explained by that the inoculation methods used in the greenhouse experiments were quite different from those in the field studies. Tubers used in the field trial were inoculated first with CIC31 and then with the biocontrol agents, while plantlets in the greenhouse studies were inoculated with the biocontrol agent first and then the pathogen. Future field and greenhouse studies will focus on identifying suitable methods for applying endophytic bacteria to seed for maximum biocontrol, and will evaluate colonization of internal plant sections by endophytic bacteria.

Table 1. Greenhouse evaluation of 114 endophytic bacteria isolated from potato.

Efficacy of endophyte as a biocontrol agent in bacterial ring rot-potato bioassay	No. strains	(%) of total
Excellent: absence of bacterial ring rot symptoms on plants after 60 days	30	26
Moderate: symptoms present an average of 3 days later than plants inoculated with CMS only	70	61
Poor: symptoms similar in all respects to plants inoculated with CMS only	9	8
Negative: symptoms present an average of 3 days earlier than plants inoculated with CMS only	5	4

Table 2. Effect of medium on *in vitro* antibiosis of *Clavibacter sepedonicum* by endophytic bacteria isolated from potatoes in Colorado.

Relative antibiosis	TSA[a]	YGM[b]	KMB[c]
-- (no antibiosis)	28[d]	48	47
+ (<0.5 cm inhibition zone)	40	31	29
++ (>0.5 cm inhibition zone)	32	21	24

[a] tryptic soy agar
[b] yeast extract glucose medium
[c] King's B medium
[d] percentage of 198 strains.

Table 3. Description and antimicrobial activity of endophytic bacteria that promoted biological control of bacterial ring rot in greenhouse trials.

Strain	Species[a]	Source	Gram Reaction	Inhibition Zone (mm) in Different Media		
				TSA	YGM	KMB
CIA40	*Enterobacter amnigenus*	cv. Sangre	neg.	3	2	2
CIA58	*Pseudomonas chlororaphis*	cv. Sangre	neg.	10	10	15
CIA82	*Enterobacter amnigenus*	cv. Russet Norkotah	neg.	7	3	0
CIA86	NO MATCH *Xanthomonas* ?	cv. Russet Norkotah	neg.	19	11	18
CIA90	*Pseudomonas chlororaphis*	cv. Russet Norkotah	neg.	4	3	2
CIA-116	*Pseudomonas fluorescens* biotype A	cv. Russet Norkotah	neg.	4	0	0

[a] species were identified on the basis of cellular fatty acid profiles by Microcheck, Inc.

Table 4. Summary of analysis of variance (ANOVA) tables for effect of strain and inoculation method on incidence of bacterial ring rot foliar symptoms in 1992.

Location	Days after planting	Strain differences	Inoculation method differences	Interactions strain x inoculation method
SLV Research Center	69	n.s.	n.s.	n.s.
"	77	n.s.	n.s.	n.s.
"	84	n.s.	** (P=.000)	n.s.
"	91	n.s.	** (P=.000)	n.s.
"	102	n.s.	** (P=.000)	n.s.
Bay Farm, Fort Collins	52	n.s.	n.s.	n.s.
"	59	n.s.	n.s.	n.s.
"	66	n.s.	** (P=.005)	n.s.
"	73	n.s.	** (P=.011)	n.s.
"	80	n.s.	** (P=.000)	n.s.
"	86	n.s.	** (P=.000)	n.s.
"	92	* (P=.019)	** (P=.000)	n.s.
"	98	* (P=.045)	** (P=.000)	n.s.
"	105	* (P=.022)	** (P=.000)	n.s.
"	110	n.s. (P=.055)	** (P=.000)	n.s.

KEY: (n.s.) = not significantly different; (*) = significant difference; (**) = highly significant difference.

Table 5. Effect of bacterial strain on populations of CIC31 detected by ELISA in stems collected from 1992 field trials.

Location[a]	Treatment (strain)	Absorbance[b]
SLV Research Center	CIC31 + CIA90	0.78 A
"	CIC31 + CIA116	0.73 AB
"	CIC31 + CIA40	0.64 AB
"	CIC31 + CIA86	0.52 AB
"	CIC31 alone	0.42 AB
"	CIC31 + CIA58	0.13 B

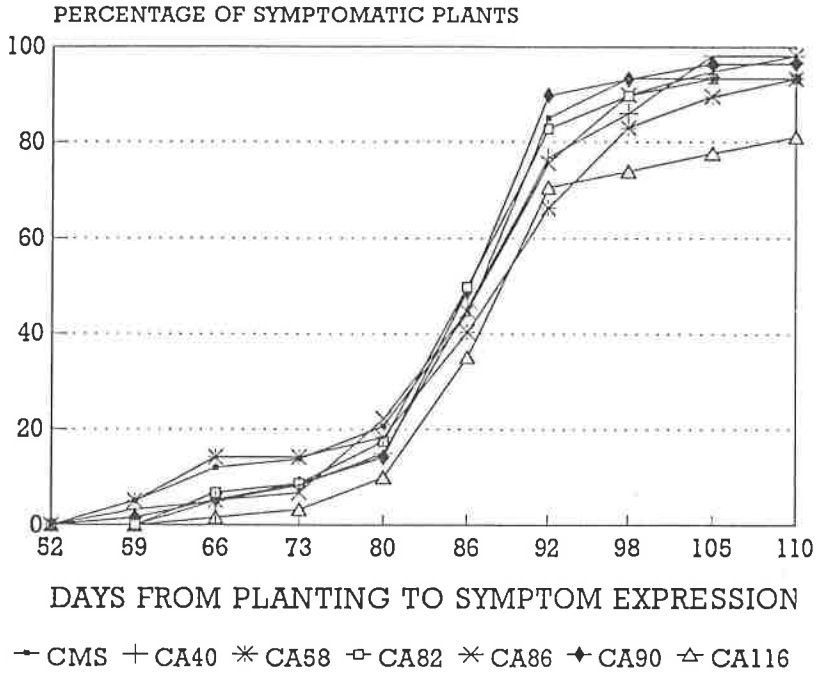
[a]Mean separations of results from the Bay Farm are not given, since mean separations by Tukey's honestly significant test were not significant, although a significant P=.045 was detected by ANOVA.
[b]Values in the same column with the same letter are not significantly different.

Table 6. Effect of inoculation method on populations of CIC31 detected by ELISA in stems collected from 1992 field trials.

Location	Knife inoculation	Vacuum infiltration
Bay Farm, Fort Collins	0.366 A	0.644 B
SLV Research Center	0.331 A	0.730 B

Key: Values in the same row with the same letter are not significantly different, according to Tukey's honestly significant means test.

A



B

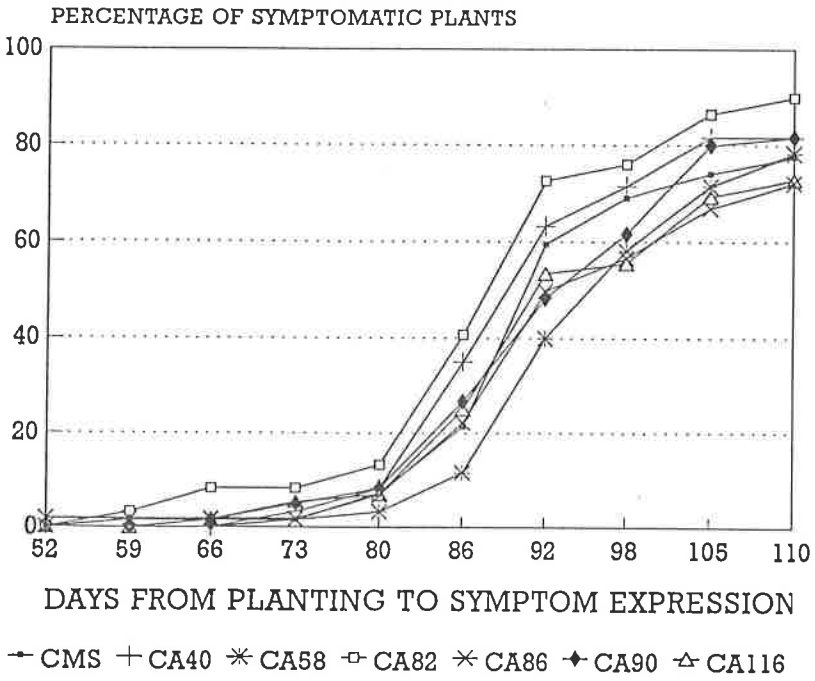
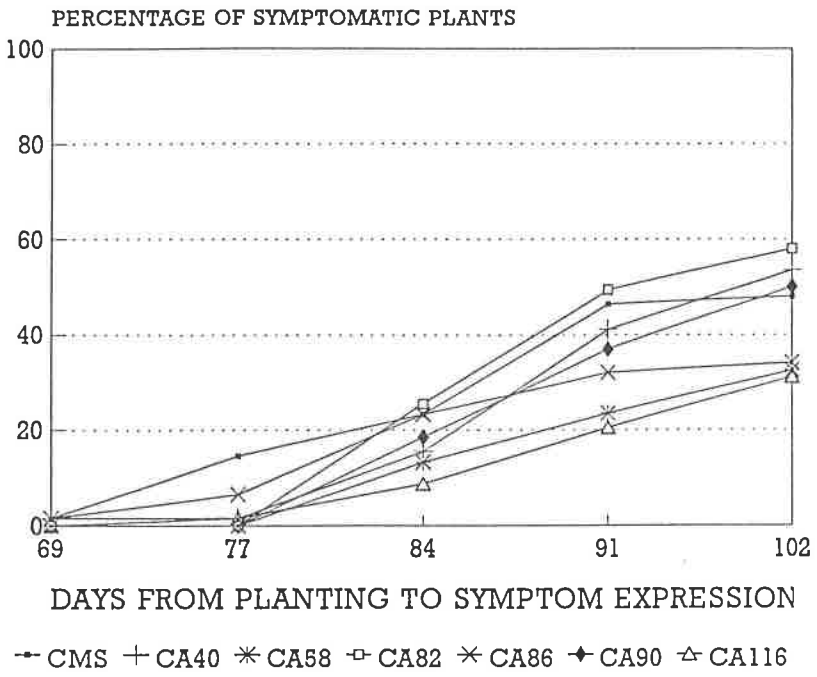


Figure 1. Effect of endophytic bacteria on foliar symptoms of bacterial ring rot from vacuum infiltrated seeds (A) and knife inoculated seeds (B) grown at the Bay Farm in Fort Collins, CO.

A



B

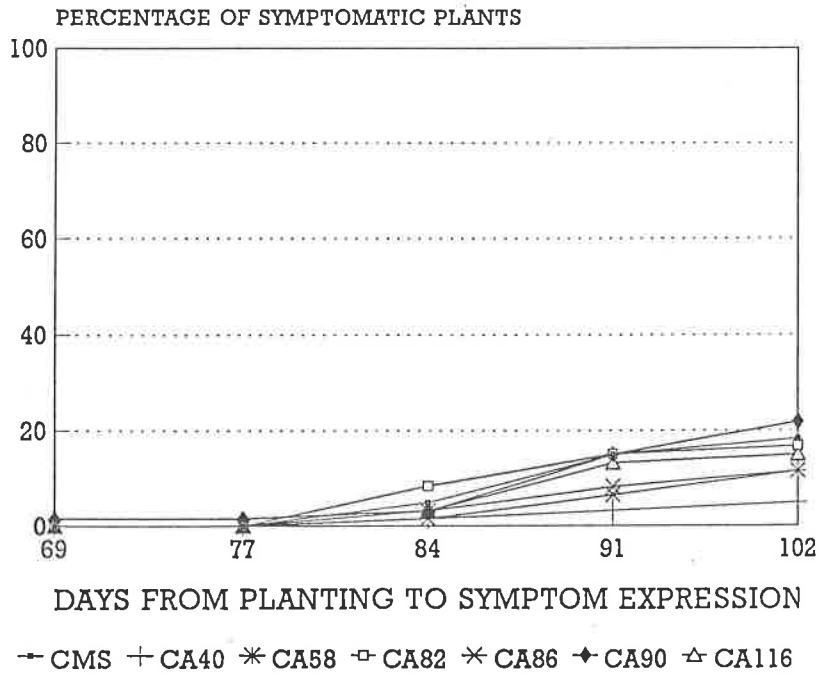


Figure 2. Effect of endophytic bacteria on foliar symptoms of bacterial ring rot from vacuum infiltrated seeds (A) and knife inoculated seeds (B) grown at the San Luis Valley Research Center, Center, CO.

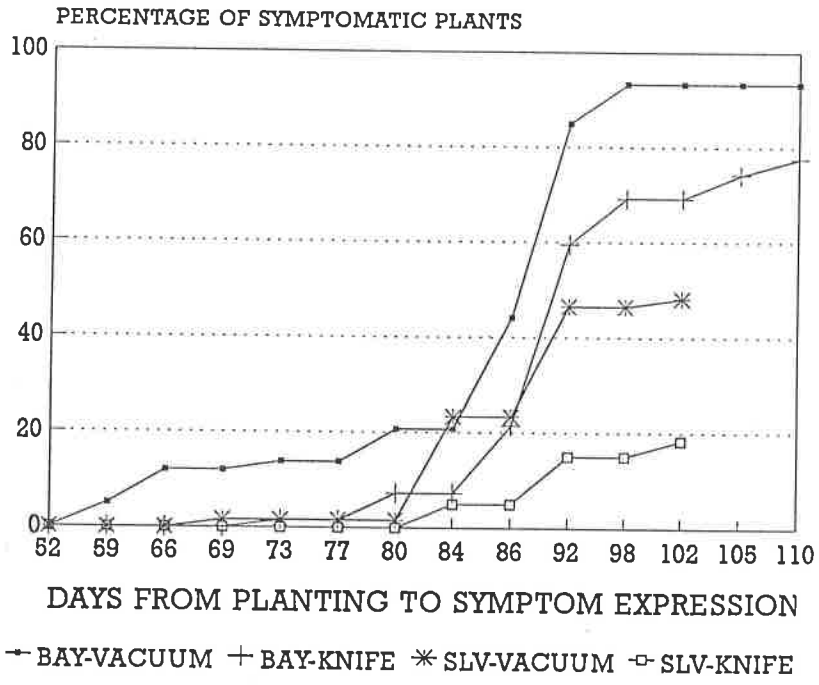
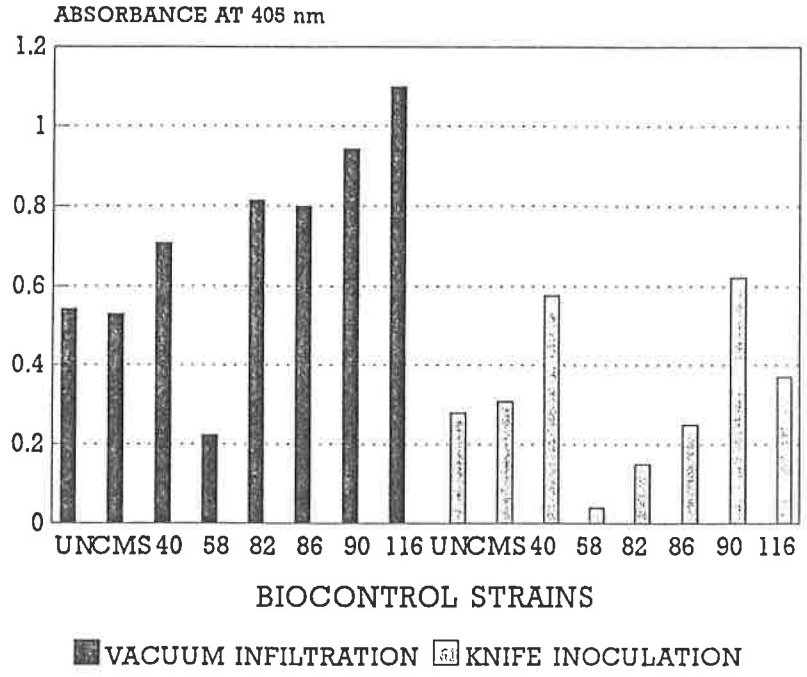


Figure 3. Comparison of foliar symptoms of bacterial ring rot associated with vacuum infiltration or knife inoculation at the Bay Farm and at the SLV Research Center, 1992

A



B

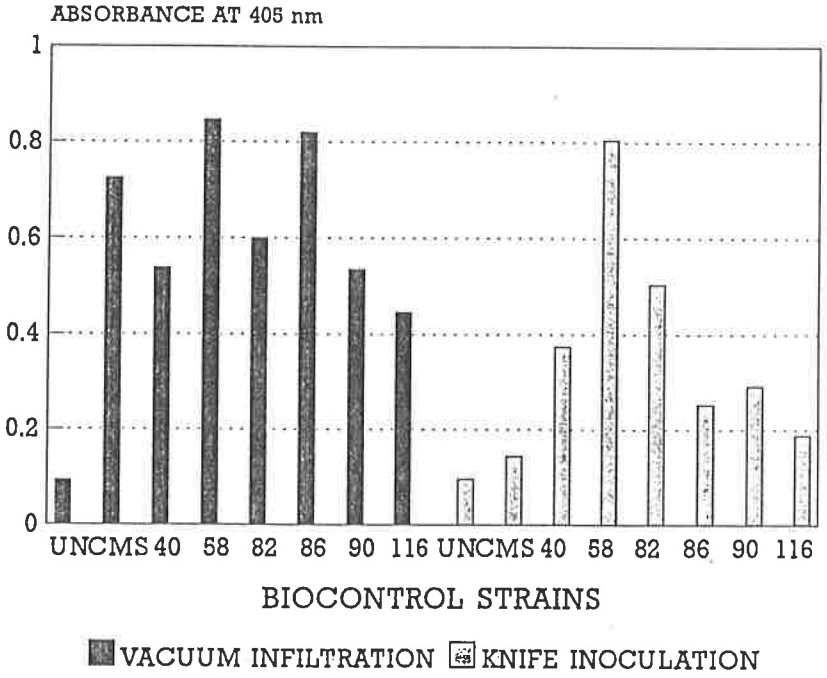


Figure 4. Effect of endophytic bacteria on CIC31 populations detected in stems from the San Luis Valley (A) or the Bay farm (B).

Objective 2: Evaluate immunity and resistance of Solanum spp. in a potato bioassay.

Introduction

Resistance to bacterial ring rot has been identified in a number of cultivars. These cultivars are omitted generally from potato breeding programs because they often delay rather than preclude symptom expression, and can harbor cell of C. sepedonicum. Although resistant cultivars have been available for a long time, the genetic basis controlling this trait is unknown. Presumably, the inhibition of symptom expression in resistant plants is a complex, quantitative trait, as shown by the strong influence of environmental factors on disease expression.

Immunity, defined as the inability of C. sepedonicum to survive and multiply in a given plant, is a type of resistance that may exist in certain wild Solanum species. Introduction of genes conferring immunity into cultivated potato varieties has been proposed as another means of eradicating the pathogen, and is being attempted through traditional breeding practices elsewhere in the United States. Presently, neither the genetic nature of immunity nor its relationship to genes conferring resistance are known.

In 1991, 1992, and 1993, Dr. Nora Lapitan and I obtained a USDA/ARS Special Agreement to use restriction fragment length polymorphism (RFLP) mapping as a technique to locate genes in Solanum spp. that confer immunity or resistance to bacterial ring rot. RFLP mapping is a technique that has proven extremely valuable for locating and identifying genes controlling important traits in humans, plants, and other organisms. RFLP mapping in plants has been used to locate genes conferring disease resistance determined by a single dominant gene, as well as genes controlling quantitative traits, such as yield. Potato is one of the few crops for which a saturated RFLP map is available. We propose that application of RFLP mapping to the potato/bacterial ringrot system will produce a rapid technique for locating the genes conferring immunity and or resistance to bacterial ring rot. A goal of our research is the production of a molecular map showing the location of genes for resistance and immunity in the chromosomes of potato. The number of genes controlling each trait and the linkage relationships of the genes will be determined. This research will provide a valuable, visual tagging method that can be used to identity plants containing immunity genes, and enable the detection of potato cultivars that may be carriers of the bacterial ring rot bacterium. The work is ongoing and requires considerable technical and hourly help and is not currently funded at an adequate level. Funds were awarded by the SLV committee in 1991 and 1992 to help support this objective as a part of my overall research program on bacterial ring rot.

To genetically map the genes for resistance or immunity to bacterial ring rot in potatoes, the most suitable genetic materials were identified first. Two criteria were established for choosing the parents for study: (a) differential response to bacterial ring rot, and (b) high degree of restriction fragment length polymorphism relative to each other. Progress on each of these aspects is presented.

Material and Methods

Tissue culture.

All plant materials are being stored as tissue-cultured clones. This provides a homogeneous supply of each accession and a source of pathogen-free plant material for the

bioassay. Much of our effort in 1991 and 1992 focussed on obtaining accessions from various sources and transferring these into tissue culture. Seeds or tubers from genetically diverse materials (Table 4) were obtained which include S. tuberosum, several diploid Solanum species, doubled monoploid lines of S. phureja, and somatic fusion hybrids between S. tuberosum and S. brevidens. Standard procedures were used to establish tissue culture stocks of each accession. All tissue culture materials arose from a single seed, tuber, or plant, to insure that tissue culture stocks of accessions represent a single, homogenous genetic background.

Screening for bacterial ring rot resistance and immunity.

The screening method used was based on the method described above to screen for biocontrol activity of endophytes. Plantlet roots are dipped into a cell suspension of C. sepedonicum strain CIC31 adjusted to an $O.D._{640}$ of 0.1-0.2, planted in pots and placed in a greenhouse for 60 days. Symptom expression is recorded, and the number of cells in each plant is estimated from viable bacterial counts monitored on antibiotic medium, and by an immunofluorescent antibody assay (IFAS).

RFLP mapping.

Leaves from greenhouse-grown plants of each accession were collected for DNA extraction and analysis of RFLP profiles. Young expanding leaves (10-20 g) were harvested and immediately frozen in liquid nitrogen. Standard methods were used to extract DNA from the leaves. The DNA was cut with appropriate restriction enzymes, separated by size on gels, and transferred to membrane filters. The blotted DNA was probed with individual DNA clones previously mapped to potato by Southern hybridization experiments.

Results and Discussion

During 1991, true seeds, tissue culture plantlets, or tubers of 53 different Solanum species were obtained from investigators in Idaho, Virginia, Wisconsin and Colorado (Table 4). A subset of these, containing 23 accessions and representing nine species of Solanum, was selected for immediate evaluation (See Table 5). To map the genes for resistance or immunity, accessions have been established in tissue culture (completed), screened for reaction to bacterial ring rot in potato (completed), and grown in a greenhouse to obtain leaves (completed) from which DNA was extracted. Southern hybridization studies to identify genetic markers that show polymorphisms at genetic loci have been completed. From these initial studies highly resistant and possibly immune accessions with DNA polymorphisms are included in the collection. From these studies, a group of accessions were selected for future breeding studies to map the gene(s) required for resistance or immunity. The F1 progeny produced will be examined in 1992-1993 for resistance to bacterial ring rot and for segregation of RFLP markers.

Table 1. Source and ploidy of accessions of *Solanum* spp. and cultivars of *S. tuberosum* used in this study.

Accession or cultivar	Source	Tissue type received	Ploidy
Solanum acaule:			
PI#210029	IR-1 Collection, Sturgeon Bay, WI	True seed	allotetraploid 2N=2X+2X=48
PI#230529	"	"	"
#6-22	C. Kriel, North Dakota State, Fargo, ND	micropropagated stem culture	"
#7-8	"	"	"
#9-7	"	"	"
S. brevidens:			
PI#CPC2451	E. Pehu, Finland	"	diploid 2N=2X=24
S. infundibuliforme:			
PI#473522-8	C. Kriel, North Dakota State, Fargo, ND (ref)	"	"
PI#473522-9	"	"	"
S. megistacrolbum:			
PI#473140-7	"	"	"
S. phureja:			
PI#320289	IR-I Collection, Sturgeon Bay, WI	true seed	diploid 2N=2X=24
PI#225678	"	"	"
#AD2-4	R. Veilleux, Virginia Polytechnic Institute, Blacksburg, VI (ref)	tubers	doubled monoploid
#AD3-4	"	"	"
#AD1-3	"	"	"
#AD29-1	"	"	"
S. sanctae-rosae:			
PI#230464-5	C. Kriel, North Dakota State, Fargo, CO	tubers	diploid 2N=2X=24
PI#230464-4	"	"	"
S. stenotomum:			
PI#458393-2	"	"	"
PI#458393-3	"	"	"

Accession or cultivar	Source	Tissue type received	Ploidy
S. tuberosum:			
Sangre S-14	Summit Enterprises, Fort Collins, CO	micropropagated stem culture	tetraploid 2N=4X=48
Sangre Std.L-1	D. Holm, San Luis Valley Research Center, Center, CO	"	"
WNC230-14	"	"	"
CO79018-11	"	"	"
Ute Russet	"	"	"
PDH40	E. Pehu, Finland (ref)	"	doubled haploid
S. verrucossum:			
PI#160228	IR-1 Collection Sturgeon Bay, WI	true seed	diploid 2N=2X=24
PI#310966	"	"	"

Table 2. Reevaluation of bacterial ring rot reaction of *Solanum* spp.

ACCESSION	PLANTS INOCULATED	SYMPTOMATIC PLANTS (%)	IFAS RATING	THIS STUDY[a]	OTHER STUDIES[b]
Solanum acaule:					
PI#210029	18	6	0.1 +- 0.1	R	R (Slack) R (Manzer)
PI#230529	6	0	2.2 +- 2.0	R	S (Manzer) R (Slack)
#7-8	12	0	0.0 +- 0.0	HR (I?)	I (Kriel)
#9-7	16	13	0.4 +- 0.7	R	I (Kriel)
S. infundibuliforme:					
PI#473522-8	12	50	3.2 +- 1.6	S	S (Kriel)
PI#473522-9	3	33	4 +-0	S	R (Kriel)
S. phureja:					S (Manzer)
PI#225678	18	6	2.3 +- 1.9	R	R (Slack)
PI#320389	8	0	2.2 +- 2.3	R	R (Manzer)
AD1-3	11	18	2.6 +- 1.9	S	not tested
AD29-1	9	0	0.0 +- 0.0	HR (I?)	not tested
S. sanctae-rosae:					
PI#230464-4	8	38	3.1+-1.6	S	S (Kriel)
PI#230464-5	10	70	2.8+-1.9	S	I (Kriel)
S. stenotomum: PI#458393-2	11	0	0.1 +- 0.2	HR (I?)	I (Kriel)
S. tuberosum:					S (Manzer)
Sangre S-14	24	79	3.8 +- 0.7	S	R (Slack)
Sangre STD				S	S (Manzer)
L-1	10	100	4.0 +- 0.0		R (Slack)
WNC230-14	11	82	2.4 +- 2.2	S	R (Manzer) R (Slack)
Ute Russet	18	11	1.0+- 1.5	R	R (Manzer) S (Slack)
CO79018-11	12	0	1.3 +- 1.6	R	R (Manzer) R (Slack)
PDH40	16	6	2.4 +- 2.0	R	not tested
S. verrucosum:					
PI#160228	16	13	2.2 +- 1.9	R	S (Slack)
PI#310966	5	20	2.0 +- 2.3	S	R (Slack) S (Manzer)

[a] S = susceptible: >15 symptomatic plants and an IFAS rating of 1-4
R = resistant: 0-15% symptomatic plants and an IFAS rating of 1-4
HR = highly resistant: 0% symptomatic plants and an IFAS rating <0.2
I = immune: 0% symptomatic plants and an IFAS rating of zero.

[b] Slack: Final research report submitted to the Plant Introduction Station, Sturgeon Bay, WI. 35pp
Manzer: Final research report submitted to the Plant Introduction Station, Sturgeon Bay, WI.
Kriel: Masters of Science Thesis, North Dakota State University, ND.

Objective 3: Compare methods for detecting and quantifying the ring rot bacterium.

Introduction

Epidemiological studies on C. sepedonicum are seriously impeded by the lack of a selective medium or an alternative method for detection and quantification of the pathogen. Most ecological studies rely on detection of the pathogen by symptom expression in an indicator plant, such as eggplant. Immunofluorescence techniques, developed by S. De Boer, and nucleic acid probes, developed by C. Orser (Idaho) and by A. Oleson (North Dakota), are available presently to identify C. sepedonicum. IFAS has also been used to quantify the pathogen in plants. As an alternative to IFAS, antibiotic-resistant mutants of C. sepedonicum were selected at Colorado State University for use in epidemiological studies. The marked strains of the pathogen are inoculated into plants and their population monitored on a medium containing the appropriate antibiotic, which enables growth of C. sepedonicum, but not indigenous microorganisms. In 1990, several antibiotic-resistant mutants were obtained from different strains of C. sepedonicum for study. The focus of studies at CSU is to verify the applicability of the various methods to detect, quantify and identify C. sepedonicum in Colorado. Validation of these methods is important to determine which methods are most applicable to seed certification and to epidemiology. The specific objectives in 1991-1992 were to evaluate the virulence of antibiotic-resistant mutants under field conditions, and to incorporate IFAS and DNA probe detection methods into the bacterial ring rot research conducted in Colorado. During these studies we became aware that unmarked and marked strains of C. sepedonicum were inconsistently recovered from plant materials and from broth cultures. Considerable effort was put into trying to figure out why C. sepedonicum would not grow consistently in our studies.

Materials and Methods

IFAS studies. Methodologies to detect single cells of C. sepedonicum in plant tissues were obtained from Carol Kriel, North Dakota. Briefly, stem samples are macerated in extraction buffer, and plant debris removed by a slow and quick centrifugation. The suspension is then recentrifuged to pellet bacterial cells, diluted ten-fold, and stored at -80C until analyzed by an

IFAS kit purchased from Agdia, Inc. Cells are viewed with a microscope equipped with a ultraviolet light source. Ten fields per well and ten wells or each dilution are viewed. Values of 0 indicate no cells per field, 2 indicates two cells per field, etc.

Effect of media. Nutrient broth yeast extract medium (NBY) was prepared with and without potassium phosphate, which is used to buffer the medium against pH changes. Cells from freshly growing plants cultures of CIC31 and its parent CIC2 were suspended in Ringer's solution of phosphate buffer, 20 mM pH7, serially diluted, and spread on NBY medium prepared with and without phosphate. Similarly, eggplant stems inoculated with CIC31 were macerated, serially diluted and spread on NBY with and without phosphate. Number of visible colonies were recorded after 7-10 days incubation at 26C.

Results and Discussion

Since 1992, my laboratory began using IFAS to detect and quantify C. sepedonicum. Preliminary experiments with pure cultures of the bacterium and infected plant materials were conducted to evaluate IFAS for screening for resistance to ring rot. The results were so encouraging that we felt confident in using IFAS as a means of screening for immunity in Solanum species. IFAS for detection of C. sepedonicum is used routinely and widely in my bacterial ring rot research.

The perplexing problem of C. sepedonicum growing inconsistently in culture was a mystery until late in 1992, when it was found that C. sepedonicum is inhibited by phosphate in NBY. Deletion of phosphate in the standard recipe for NBY increased recovery of C. sepedonicum dramatically (Table 1). We plan to evaluate this phenomenon further, as it may aid in the development of improved culture media with which to conduct epidemiological studies on C. sepedonicum.

Table 1. Effect of phosphate on growth of CMS in NBY media.

	NBY		NBY + Rif^{50}	
Strain	with PO_4	w/o PO_4	with PO_4	w/o PO_4
CIC2 (rif^S)	$< 10^5$	4 X 10^7	0	0
CIC31 (rif^R)	$< 10^5$	2 x 10^7	$< 10^5$	2 X 10^7
CIC31 (rif^R) from potato	$< 10^6$	1 x 10^9	$< 10^6$	1 x 10^9

[a] values represent colony forming units per ml (cfu/ml).