SUMMARY RESEARCH PROGRESS REPORT FOR 1990 AND RESEARCH PROPOSAL FOR 1991

Submitted to: SLV Research Center Committee
and the Colorado Potato Administrative Committee (Area II)

Title: Innovative strategies for the detection and control of bacterial ringrot

Project Leader: C. A. Ishimaru, Department of Plant Pathology and Weed Science

Project Justification:

Bacterial ringrot 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 ringrot 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, availability and use of disease free seed can not eradicate the pathogen, Clavibacter sepedonicum, which is often present in the absence of disease symptoms. There are no chemical control measures for eliminating the pathogen in seed used in table stock production, or for preventing certified seed from becoming contaminated by any of several possible sources identified by recent research. Chemical disinfestation of cutting and handling equipment and storage facilities help limit spread, but are inadequate methods for preventing recontamination by bacteria carried in irrigation water or on weeds. The primary objective of this proposal is the development of innovative strategies for the detection and control of bacterial ringrot.

Project Status:

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. This year I want to continue the biological control studies and also add projects that would lead to the ultimate goal of managing bacterial ringrot. One of these objectives concerns the early detection of the pathogen using new techniques developed elsewhere in the country. Such techniques could be incorporated eventually into Colorado's seed certification program. Another objective focuses on identifying the molecular basis of latency of bacterial ringrot in potato by identifying genes in potato that are responsible for resistance and or immunity to the disease. Information from these studies could lead to easy screening of potato clones for the presence of either desirable or undesirable resistance genes. I have received funds from the USDA to investigate the use of restriction fragment length polymorphism (RFLP) mapping to identify genes conferring resistance to ringrot in potato. This is a joint project with Dr. Nora Lapitan from the Department of Agronomy. All of these projects require extensive laboratory and greenhouse testing before field evaluations can begin. Field testing of biocontrol agents could begin in 1992, depending on results from greenhouse studies. I have changed the name of this year's proposal to reflect the broader interests of my program.

Soon after starting my studies, I realized the need for a bioassay system that could be used under controlled environmental conditions, and still provide information applicable to the field. I chose to develop a potato bioassay in which tissue-cultured plantlets of potato are the source of susceptible or resistant plants. Potato is the natural host for C. sepedonicum and results are likely to mimic what happens in the field. Eggplant has historically been used as an indicator plant in greenhouse studies, because symptoms are observed in a reasonable time after inoculation with the pathogen. In contrast, symptoms have rarely been observed in inoculated potatoes grown from seed pieces and planted in a greenhouse; however, Schuld and Harrison found that tissue-cultured plantlets of potato readily express symptoms within 30 days after inoculation. We have confirmed these findings and can consistently obtain symptom expression in tissue-cultured potatoes. To monitor bacterial populations in the bioassay, I have obtained antibiotic-resistant

strains of C. sepedonicum. Antibiotic-resistant strains of plant pathogenic bacteria have been used in several other plant-pathogen systems to gain information on population size of a pathogen and its role in plant disease development. Bacteria marked with antibiotic resistance can be distinguished from other bacteria in plant materials by their growth on media containing the appropriate antibiotic. The potato bioassay and antibiotic-resistant mutants we have developed will be used for many different aspects of bacterial ringrot research, including biological control, detection, and latency studies.

Significant Accomplishments 1990-1991

A major accomplishment for the year was the development of the potato bioassay and antibiotic-resistant mutants for bacterial ringrot studies. All of the equipment and supplies required for tissue-culture propagation of potatoes have been purchased. Potato plantlets of the cultivars Sangre, Russet Burbank, Centennial Russet and Ute have been propagated. Eight different methods for inoculating plantlets with the ringrot bacterium have been evaluated. Preliminary results indicate that inoculations through root dips and stem injections yield the most consistent results. We have obtained antibiotic-resistant mutants of several isolates of C. sepedonicum. Strains of the pathogen were isolated from infected tubers and stems collected in Colorado, and spontaneous mutants were selected for their resistance to either streptomycin or rifampicin. Growth of these mutants in plants is being compared to the parents. Preliminary results indicate some mutants and parents grow at different rates in plants.

Significant progress in the biological control work has also been made. A collection of 769 bacteria was obtained from the vascular sap of potato stems collected throughout the 1990 growing season from fields in the San Luis Valley. Sap from diseased and healthy plants was spread onto King's B medium, nutrient broth-yeast extract medium (NBY) and NCP-88, a semi-selective medium for C. sepedonicum. Two isolates from each plate were selected at random, purified, and stored at -80C in glycerol. The next step will be screening for biocontrol activity in the potato bioassay.

Objectives for 1991:

1) Continue work on the biological control of bacterial ringrot. We will screen the endophytic bacteria collected in 1990 for biocontrol activity in the potato bioassay. We will also screen strains for in vitro antibiosis. 2) Evaluate immunity and resistance of Solanum sp. in the potato bioassay. This is the first step in identifying useful plant materials for future cloning and characterization of resistance and immunity genes from potato. Seeds of Solanum sp. will be obtained from other scientists in the United States, who have evaluated the accessions for their immunity or resistance to ringrot. Each accession's reaction to ringrot will be confirmed in the potato bioassay. 3) Compare methods for detecting and quantifying the ringrot bacterium. We will evaluate the sensitivity and specificity of a new DNA probe and polymerase chain reaction (PCR) developed in Idaho by Dr. C. Orser, and an immunofluorescent serological detection assay (IFAS) developed by Dr. S. DeBoer. These studies will be conducted in the potato bioassay using antibiotic-resistant strains of the pathogen.

Funding:

1990-1991 Allocation	$11,500
1991-1992 Request	$12,000

Budget Summary:

Labor	$ 8,500
Supplies	$ 3,000
Travel	$ 500
Total	$12,000

Annual Report

Selection of Biocontrol Agents Antagonistic Toward the Ringrot Bacterium

Submitted by:

C. A. Ishimaru
Department of Plant Pathology and Weed Science
Colorado State University

Abstract

A collection of 769 bacteria was obtained from the vascular sap of potato stems collected throughout the 1990 growing season from fields in the San Luis Valley and elsewhere. A major accomplishment for the year was the development of a potato bioassay and antibiotic-resistant mutants that can be used to measure biological control activity of the bacteria in the endophyte collection. Ten inoculations methods were tested. Micropropagated plantlets of the cv. Sangre uniformly expressed BRR symptoms after soaking trimmed roots of the plantlets in cell suspension of the pathogen. Drenching the soil with the pathogen was the least efficient inoculation method tested. Comparisons between parental strains and antibiotic-resistant mutants were measured in an eggplant bioassay. Preliminary results indicate that resistance to antibiotics can affect BRR disease severity in eggplants.

Introduction

Bacterial ringrot (BRR), caused by Clavibacter sepedonicum, remains a serious concern to the commercial and seed potato industry in Colorado. There is a definite need to develop control

strategies that can augment current cultural and sanitation practices for managing BRR. As with many other bacterial disease, there are no effective agrichemicals available to control BRR. Biological control of plant diseases through the application of nonpathogenic microorganisms has been proposed for other bacterial diseases of potato. Biological control of BRR has not been attempted previously, but several observations suggest it's feasibility. Many microorganisms inhibit the growth of C. sepedonicum in vitro, and make isolation of C. sepedonicum from plant materials difficult. In 1972, De Boer reported the presence of large numbers of diverse, nonpathogenic bacteria in the vascular system of stems from healthy potato plants. The ecological significance of such "endophytic" bacteria in potato remains unknown. It appears, however, that the vascular system of potato is an ideal niche for many types of microorganisms. An important consideration for future implementation of biological control is it's practicality. Application of biological control agents to potatoes is practical, because biocontrol agents can be applied directly to seed pieces prior to planting. Seed piece inoculations have been used successfully to apply bacterial antagonists for the control of potato soft rot diseases. If biological control was shown to be effective, commercial potato production would benefit immediately. The value and usage of biological control agents in certified seed production may be less, and would depend on their effect on BRR expression.

A primary objective of my research is to evaluate the potential efficacy of biological control agents for BRR. To accomplish this objective I developed a potato bioassay in which endophytic bacteria can be evaluated for biological control activity. Potato is the natural host for C. sepedonicum and results are likely to mimic what happens in the field. Eggplant has historically

been used as an indicator plant in bioassays because ringrot symptoms are observed in eggplant within a reasonable length of time (14-45 days). In contrast, BRR symptoms are rarely observed in inoculated potatoes grown from seed pieces and planted in a greenhouse. Recently, however, B. Schuld and M. D. Harrison demonstrated that tissue-cultured plantlets of potato express symptoms of ringrot within 7-30 days after inoculation with C. sepedonicum. This suggested that tissue-cultured plantlets of potato may be suitable for measuring biological control activity. The following is a summary of work completed to date on development of a potato bioassay and biological control agents for BRR.

Materials and Methods

Bacterial strains.

Strains of C. sepedonicum were obtained from other culture collections and from diseased potatoes collected in Colorado. Potato stems were collected at three different times during the 1990 growing season from four locations in the San Luis Valley. Plant extracts were spread onto NCP-88, a semi-selective medium for isolating C. sepedonicum, and the cells incubated for 7-10 days at 26 C, after which time small, pinpoint colonies typical of the pathogen were observed. Individual colonies were purified and stored in glycerol at -80C. Identification of isolates as C. sepedonicum was verified by an immunological test (ELISA) specific for C. sepedonicum. Pathogenicity of each isolate was confirmed by inoculating strains into eggplant and observing typical ringrot symptoms.

Bacteria to be tested for biological control activity were isolated from the stems of healthy

and BRR infected plants. Stems were rinsed in distilled water and surface sterilized by dipping in alcohol and flaming. A one centimeter section of the basal portion of each stem was aseptically removed and soaked in 1 milliliter of phosphate buffer for 1-2 hours. Dilutions of the samples were spread onto the surface of three agar media: King's B medium, nutrient broth-yeast extract medium (NBY) and NCP-88 medium. Cells were incubated at 26C for 2-3 days on Kings' B and NBY or 6 days on NCP-88 medium. Two isolates from each plate were selected at random, purified by selection for single colonies 2-3 times, and stored at -80C in glycerol.

All bacteria were recovered from frozen glycerol stocks by streaking a small chunk of the culture onto an appropriate medium and incubating for 2 days (endophytes) or 5 days (C. sepedonicum) at 26C. Subculturing of strains was kept to a maximum of two transfers, including the first transfer from glycerol.

Selection and characterization of antibiotic-resistant mutants.

Spontaneous antibiotic-resistant mutants of C. sepedonicum were obtained by spreading about 10^{10} cells of each strain onto antibiotic-amended medium and incubating for 10-14 days at 26C. Three different media were tested as the base medium for selection of mutants to rifampicin or streptomycin at 50 ug/ml. A nutrient broth-yeast extract medium (NBY) yielded the greatest number of mutants to either antibiotic, and was consequently used in all subsequent experiments. Colonies were selected from three different selection plates. Each mutant was purified by single colony isolations and stored in glycerol at -80C.

Characterization of in planta growth of selected antibiotic-resistant mutants was measured in an eggplant bioassay. Five week-old eggplants (cv. Black Beauty) were inoculated with 10 ul of each strain adjusted to about 0, 10^4, 10^6 and 10^8 cfu/ml in phosphate buffer (20 mM, pH7), corresponding to inoculum doses of 0, 10^2, 10^4, and 10^6 cells, respectively. Plants were grown in a greenhouse for 40 days under 16 hours light, 8 hours dark. Temperatures in the greenhouse varied between 20-28 C. Peter's Professional fertilizer (20-20-20 NPK) was applied routinely to promote healthy, vigorous growth. Plants were observed daily for symptoms. The experimental design was a randomized block with four replications and three plants per pot. Statistical analyses were performed using analysis of variance procedures in SAS and SPSS statistical packages.

Potato bioassay.

Micropropagation of potato plants was accomplished using the procedures recommended by the Colorado Seed Certification Program. Culture stocks of several potato cultivars were kindly provided by Rob Davidson. Sangre plantlets were obtained from Summit Enterprise, Fort Collins, CO. Plantlets were multiplied by stem cutting micropropagation and grown for about 6-8 weeks in a growth chamber set at 16 hours light at 24C and 8 hours light at 18C.

Prior to inoculation with C. sepedonicum, plantlets were hardened in a greenhouse by opening the lids of each culture jar for 1-2 days. Plantlets were gently removed from the culture jar and rinsed briefly with sterile distilled water to remove any adhering agar. After potting in 10.2 cm pots in TerriLite Metro Mix 352, plantlets were grown briefly under shade by hanging a black screen over the bench for one week to continue hardening. Several inoculation methods

were tested (see Table 2). The strain of C. sepedonicum used in all of these tests was CIC2 adjusted to about 10^8 cfu/ml in buffer. Greenhouse conditions and fertilizing schedules were the same as described above for the eggplant bioassay. Forty-one days after planting, a 1 centimeter section, from the soil line up, of each stem was aseptically removed and analyzed by ELISA for the presence of C. sepedonicum.

Results

Isolation of pathogenic strains of C. sepedonicum.

Strains of C. sepedonicum were readily isolated from infected plant materials by culturing extracts onto NCP-88 medium. Very dilute samples of highly contaminated materials plated onto NCP-88 yielded the greatest number of colonies of C. sepedonicum. At more concentrated dilutions these same samples yielded few colonies of C. sepedonicum. Antagonism of C. sepedonicum by the other endophytic bacteria that grew on NCP-88 medium was obvious in the concentrated samples. Nineteen strains of C. sepedonicum were isolated from potatoes grown in Colorado. An additional eight strains were obtained from other culture collections. All strains were virulent in eggplant, grew on NCP-88 and were positive for C. sepedonicum in ELISA tests.

Isolation and characterization of antibiotic-resistant mutants of C. sepedonicum.

Selection of spontaneous mutants of C. sepedonicum resistant to the antibiotics streptomycin or rifampicin was completed. Frequency of mutations to rifampicin or streptomycin resistance were 10^{-9} and 10^{-10}, respectively. Another antibiotic, nalidixic acid, was not useful as a marker,

because all isolates of C. sepedonicum tested were naturally resistant to nalidixic acid at 25 ug/ml. A high degree of background resistance to nalidixic acid in the microflora isolated from potato was evident by the growth of numerous (10^4-10^5 colony forming units/ml) bacteria on NCP-88 medium, which contains nalidixic acid as a selection for C. sepedonicum. A total of five to ten antibiotic-resistant mutants from each of six Colorado and seven other isolates of C. sepedonicum were obtained for each of the two antibiotic classes.

In planta growth of three parental strains of C. sepedonicum from Colorado and 12 of their corresponding mutants was evaluated in the eggplant bioassay. Days to symptom expression, percentage of leaves with symptoms, and the number of plants showing symptoms/ three plants/ pot were recorded (Figures 2, 3, and 4). Two mutants in each antibiotic resistance class per strain were analyzed. Statistical analyses of these data is in progress and it remains to be determined if the difference seen in Figure 2, 3, and 4 are statistically different. However, the data in Figures 2, 3, and 4 strongly suggest that certain mutants behave differently than their wild type strain (parent) in planta.

Endophytic bacteria.

Endophytic bacteria were isolated from all potato stems collected during 1990. Different colony types were observed on each of the three media used for selection. The purified cultures varied dramatically with respect to growth rate and colony morphology, indicating a broad range of bacteria were isolated by our method. Table 1 summarizes the sample dates and field descriptions used to obtain the endophyte collection. A total of 736 strains from SLV and 33

strains from other sources were isolated and stored.

Potato bioassay.

Various inoculation methods were tested to determine the best method for obtaining consistent BRR symptoms in greenhouse grown potato plantlets. A summary of the effect of inoculation methods on the number of days before symptoms were observed in plantlets of Sangre is presented in Table 2 and Figure 1. Increasing the length of time that roots were soaked in cell suspensions of C. sepedonicum resulted in a decrease in the number of days before symptoms were observed and a smaller standard deviation. Drenching the soil with inoculum was less effective, but disease was obtained with this method.

The presence of BRR symptoms correlated well with ELISA results. Of the 78 stems analyzed from symptomatic plants, 76 tested positive in ELISA. Twenty one symptomless plants were also tested. Nine of these tested positive and six gave weakly positive reactions in ELISA. Six of the symptomless plants had negative ELISA reactions, and four of these were from treatment 8, which had the fewest symptomatic plants at the time of harvest. These results show that the symptoms observed were caused by C. sepedonicum.

Other micropropagated cultivars of potato also expressed symptoms of BRR after inoculation with C. sepedonicum. Plantlets of Russet Burbank, Centennial Russet and Ute Russet were inoculated with C. sepedonicum by soaking the trimmed roots of each plantlet for five minutes in a cell suspension of CIC2. Three plants per cultivar were inoculated. Controls were

inoculate with phosphate buffer only. Symptoms of BRR in Russet Burbank were visible after 31-36 days, and after 38-39 days in Centennial Russet plants. Symptoms were not seen in Ute Russet even after six weeks. No BRR symptoms were observed in control plants.

Discussion

We have developed an easy and reproducible method for obtaining uniform symptom expression of BRR in greenhouse-grown potato plants. The method has been evaluated most extensively with cv. Sangre, but preliminary evidence indicates that other cultivars also work well in the bioassay. The easiest and most efficient inoculation method tested is a root soak for 20 minutes preceded by a brief drying period. This method of inoculation resulted in symptom expression after an average of 29.7 days. Plantlets inoculated in this manner uniformly expressed BRR symptoms within a few days of each other (standard deviation of 2.26 days). Similar symptom expression data was also obtained by inoculating stems by removing upper leaves of plantlets before immersing in inocula; however, this method is more laborious and time consuming than the root dip. Symptoms observed in the potato bioassay were typical of BRR and included marginal wilting of the lower leaves followed by slight chlorosis and necrosis of the affected leaves. Interveinal chlorosis started to appear at about 40 days after planting, but, because plants were harvested at 41 days, it is not known whether or not the symptoms would have advanced further. I plan to verify these results by replicating the experiment.

To prevent the loss of virulence of the pathogen, previous BRR studies conducted in

Colorado used, as a source of inoculum, cells of C. sepedonicum that had been stored in plant materials This procedure is very laborious and has a high probability of producing mixed cultures of the pathogen. Currently, a common procedure for long term storage of plant pathogenic bacteria is storage of strains in glycerol at -80C. We routinely store other bacteria in this manner and found this to be a very suitable storage method for C. sepedonicum. All strains of C. sepedonicum stored in glycerol at -80C retained viability and were highly virulent in eggplant bioassays.

Antibiotic-resistant strains of plant pathogenic bacteria have been used in several other plant-pathogen systems to gain information on population size of a pathogen and its role in plant disease development. Bacteria marked with antibiotic resistance can be distinguished from other bacteria in plant materials by their growth on media containing the appropriate antibiotic. Populations of a marked strain can be monitored over time on or in individual plant parts inoculated with the marked strain. Differential growth rates of the pathogen in resistant versus susceptible cultivars can also be determined. In plant and in vitro growth of antibiotic-resistant mutants should be compared to wild type strains before mutants are used extensively as research tools. The data presented in Figures 2, 3, and 4 indicates the need to evaluate antibiotic-resistant mutants of C. sepedonicum before proceeding with them in other experiments. Though statistical analyses and replication of the experiment is needed to confirm the similarity or dissimilarity of mutants and parents, these preliminary data indicate that some of the mutants would not be predicated to behave as the wild type strains in planta. Evaluation of two other strains and their antibiotic-resistant mutants is currently in progress. In the end, I plan to have evaluated antibiotic-

resistant mutants from eight strains of C. sepedonicum, six of which were isolated from potatoes grown in Colorado. If none of these mutants are determined to be suitable for future studies, other mutants in the collection will be screened. (Only two of the possible 5-10 mutants in our collection for each antibiotic class and strain will have been tested). During the 1991 growing season, some of these mutants will be evaluated in field trials conducted in collaboration with G. Franc, K. Knudson, and R. Davidson.

Screening for biological control activity among the endophytes collected during 1990 was not possible because of the lack of an appropriate assay for measuring their activity under controlled conditions. Now that a potato bioassay has been developed, screening the endophyte collection can begin. Another way to assess the potential biological control activity of an endophyte is to look for its ability to inhibit the growth of C. sepedonicum in culture (in vitro). However, in vitro antibiosis is not always correlated with biocontrol activity in planta. In vitro antibiosis could be useful when considered together with results from a potato bioassay. Since the methods for antibiosis screening are relatively simple compared to those of a plant bioassay, most of my research has focused on the development of the latter, with the intention of conducting in vitro antibiosis experiments as soon as an adequate plant assay was available.

The potato bioassay I have developed will be used for many aspects of bacterial ringrot research. To my knowledge, a potato bioassay for studying bacterial ringrot is under development in only two other laboratories in North America. Together with the antibiotic-resistant mutants, we now have a system in Colorado for investigating biocontrol and latency of bacterial ringrot.

Furthermore, the potato bioassay will provide a means of identifying the genetic basis of resistance and immunity to bacterial ringrot in potato through restriction fragment length polymorphism (RFLP) mapping.

Table 1. Description of sites sampled in 1990 for collection of endophytic bacteria.

Sample Date	Field Description	Number of stems collected	Number of endophytes collected
July 16	Sangre G-4 no BRR present	10	60
" "	Russet Norkotah NGC no BRR present	10	60
" "	Sangre 11 no BRR present	10	60
" "	Russet Nugget no BRR present	10	60
" "	Commercial lot cultivar unknown no BRR present	10	44
August 3	Sangre BRR present; healthy plants collected	10	56
" "	FritoLay 795-1 no BRR present	10	58
" "	Russet Norkotah no BRR present	10	53
" "	Russet Norkotah no BRR present	10	60
August 27	White Rose BRR present	10 healthy 9 diseased	60 18
" "	Centennial Russet BRR present	10 healthy 13 diseased	67 26
" "	Centennial Russet no BRR present	10	54
July 24 (Gilcrest)	Norgold Russet no BRR present	1	8
" "	Sangre no BRR present	5	24
" "	Corn plant with Goss' wilt	1	1

Table 2. Effect of inoculation method on expression of BRR symptoms in micropropagated potato plantlets.

Treatment number	Description	Proportion of plants with BRR symptoms	Average number of symptomless days
1	roots trimmed, soaked immediately for 5 minutes	7/10	33.70 ± 5.33
2	roots trimmed, soak immediately for 10 minutes	8/9	31.10 ± 4.12
3	roots trimmed, soak immediately for 20 minutes	10/10	30.20 ± 3.39
4	roots trimmed, wait 10 minutes, soak for 10 minutes	8/10	33.30 ± 5.21
5	roots trimmed, wait 10 minutes, soak for 20 minutes	10/10	29.70 ± 2.26
6	lower leaves trimmed, soak stem 10 minutes	10/10	29.20 ± 1.75
7	upper leaves trimmed, soak stem 10 minutes	9/10	32.30 ± 3.56
8	stem injected with 10 ul cells, immediately after planting	9/10	29.60 ± 4.22
9	stem injected with 10 ul of cells, two weeks after planting	1/10	40.70 ± 0.95
10	soil drench	6/10	35.80 ± 4.61

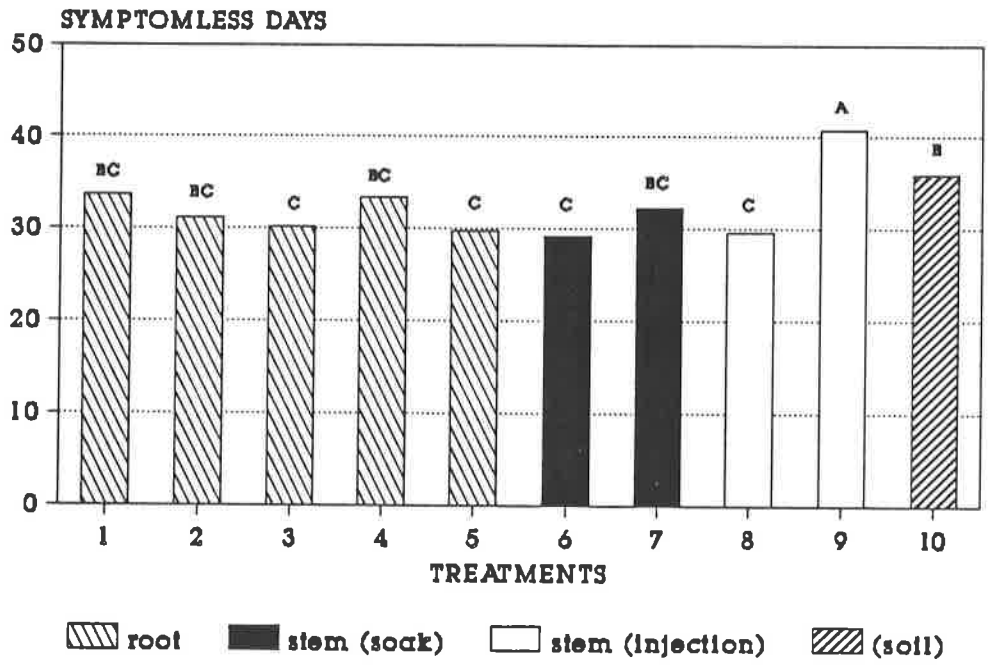

Figure 1. Average number of symptomless days observed in micropropagated potato plantlets of cv. Sangre inoculated by various methods with cells of C. sepedonicum. Treatment descriptions for each category (root, stem soak, stem injection, and soil) correspond to those listed in Table 2.

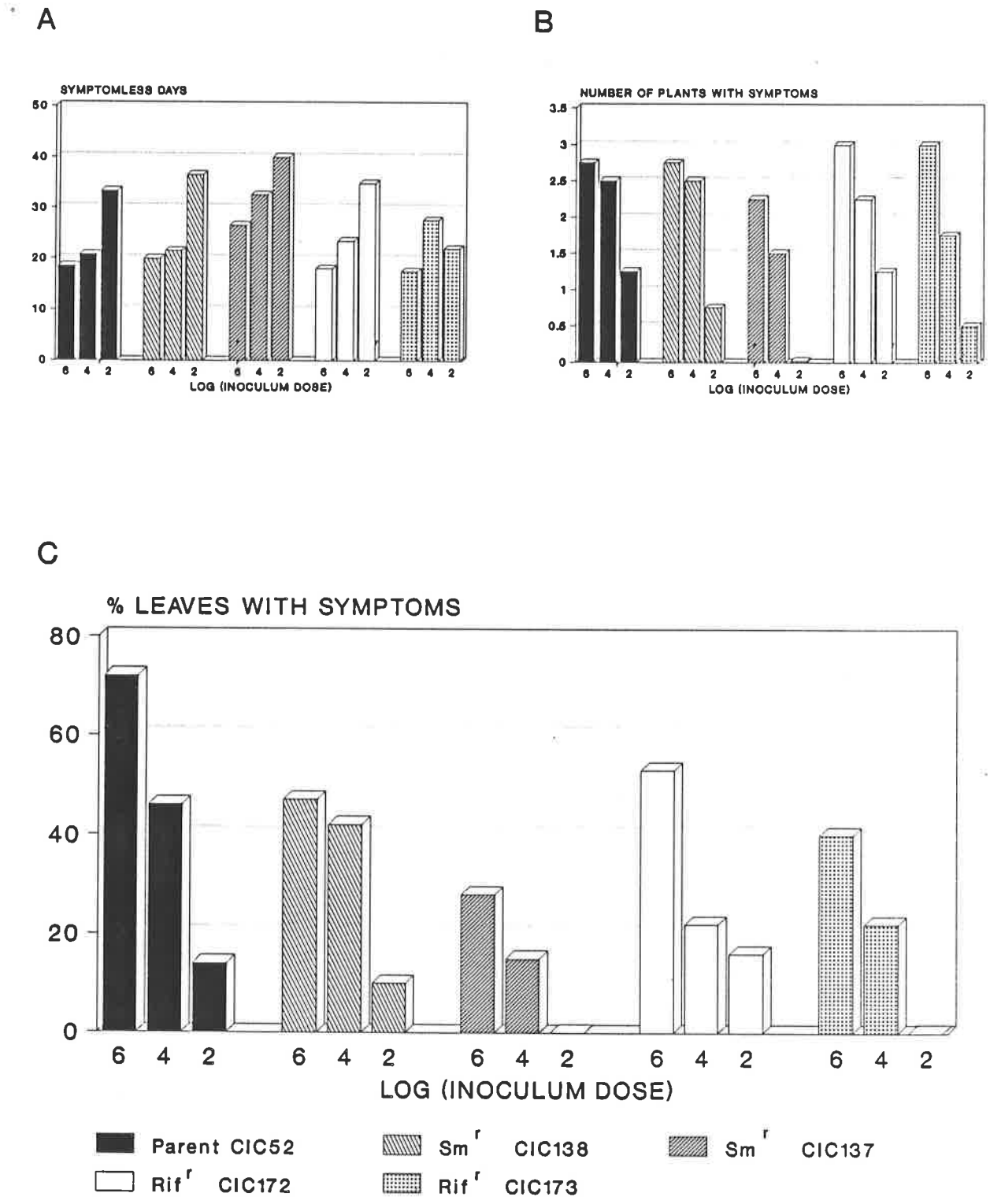

Figure 2. CIC52 and its derivatives in eggplant bioassay.

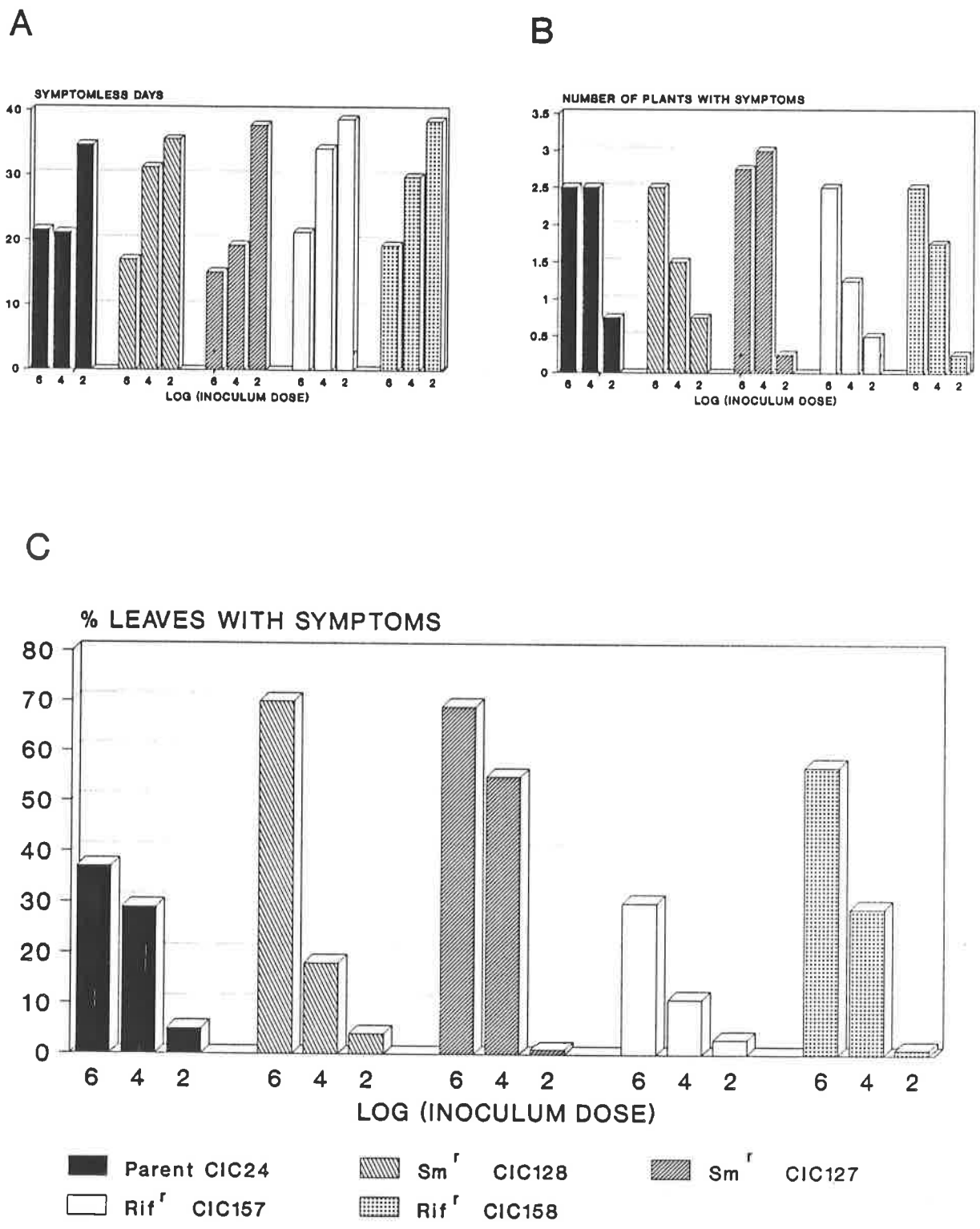

Figure 3. CIC24 and its derivatives in eggplant bioassay.

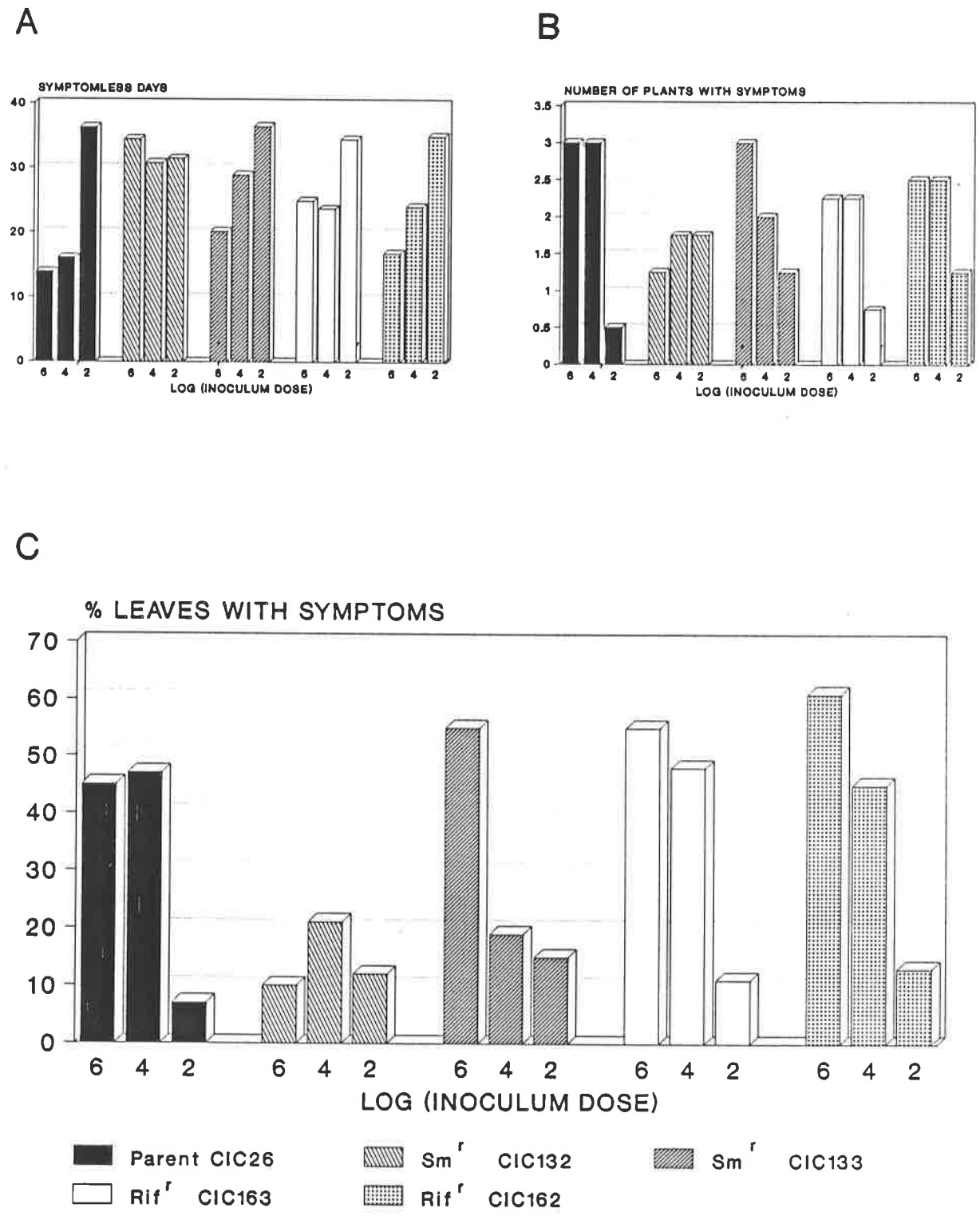

Figure 4. CIC26 and its derivatives in eggplant bioassay.