FINAL REPORT

Covering period 1992 - 1998

Submitted to the Office of the Science Advisor
U S Agency for International Development

Survival of Plant Pathogenic Bacteria in Tropical Soils Traced with Monoclonal Antibodies

Principal Investigator Anne M Alvarez
Grantee Institution University of Hawaii
College of Tropical Agriculture and Human Resources

Collaborator Dilli D Shakya
Central Department of Botany, Tribhuvan University
Kitipur, Nepal

Project Number 12 691
Grant Number 936 5600
(HRN-5600-G-00-2009-00)
A I D Grant Project Officer Dr John Daly
now Andres Orange

Project Duration August 1, 1992 to July 31, 1998

BEST AVAILABLE COPY

TABLE OF CONTENTS

I	Executive Summary	1
II	Research Objectives	2
III	Methods and Results	5
IV	Impact and Relevance of Technology Transfer	12
V	Project Activities, Output	14
VI	Project Productivity	15
VII	Future Work	16
VIII	Literature Cited	17
IX	Appendix I Figures and Tables	20
X	Appendix II Attachments (papers and abstracts)	25

I EXECUTIVE SUMMARY

A research program sponsored by USAID, was carried out to understand the survival of a bacterial pathogen, *Xanthomonas campestris* pv *campestris*, in soil and plants in tropical and subtropical environments and to erradicate the devastating effects of black rot of crucifers in Hawaii and Nepal The work has been performed by a joint effort of Tribhuvan University (TU) of Nepal and the University of Hawaii (UH) in USA New methodology for detection of *X campestris* pv *campestris* (Xcc) was developed Epidemiology and perpetuation of disease were studied with a selected panel of monoclonal antibodies (MAbs) Farm management practices were designed in order to minimize black rot damage

Strains of Xcc were isolated from soils, plants and seeds from Nepal and Hawaii and classified with MAbs For epidemiological and survival studies a virulent strain of Xcc was bioengineered to contain the 'lux' gene to make it bioluminescent, permitting quantitative nondestructive detection of live Xcc cells in soil or leaf tissues The influence of moisture regimes of soils and competition with other bacteria on the phylloplane of crucifers and rotational crops were related to survival of Xcc The role of plant nutrition on infection rates and disease development was determined In all cases, the pivotal and imprescindible tools for monitoring Xcc populations were specific MAbs and the Xcc "lux" strain

The tranfer of information and training has taken considerable effort and is a major part of the accomplishments of the project Faculty and trainees from TU visited Hawaii on several occasions in order to learn technical skills and to assimilate the advances of the project Dr Dilli D Shakya, our collaborator in the Central Department of Botany in Tribhuvan University, came to Hawaii on three occasions in order to participate in the "technology transfer' on immunodiagnostic tests and survival studies Two graduate students from Nepal and three from Hawaii participated in the project, and their work resulted in one MS thesis and two Ph D dissertations The students mastered the techniques using serology and bioluminescent markers for survival studies The P I and project director visited Nepal, surveyed the production sites and offered workshops and training at TU A technical seminar was given at the USAID headquarters in Kathmandu followed by a round-table discussion on strategies for improvement of crucifer production in Nepal Participants included invited guests from the Ministry of Agriculture government research and extension personnel, faculty and graduate students from Tribhuvan Univesity and the USAID team in Nepal In conclusion, 1) Sufficient knowledge is now available to adapt better disease management for crucifers in Nepal and with adoption of appropriate cultural practices losses due to black rot can be substantially reduced, 2) Findings of laboratory, greenhouse, and field research lead to changes in management procedures, some of which are common to both Hawaii and Nepal More progress, particularly in Nepal, will be achieved if appropriate detection and identification methods are used to solve the major problem, that is, detecting infected seed and making clean seed available to farmers 3) Seventeen publications (refereed articles, short articles theses, abstracts and bulletins) resulted from the project The two bulletins were prepared as instructional materials for national scientists and extension personnel These publications could also be used to extend the results to other developing countries

II RESEARCH OBJECTIVES

A Why was the project conducted? Black rot disease is the most devastating and economically important disease of crucifers worldwide Disease management strategies are based on thorough knowledge of pathogen disssemination spread and survival, but little is known about the epidemiology of the disease and/or the survival of the pathogen in tropical and subtropical environments where edaphic factors and cultural practices are so unlike those of temperate zones Crucifer production practices in Hawaii has much in common with practices in Nepal where land is usually recropped without adequate fallow, promoting the build-up of inoculum and the perpetuation of plant disease When conditions for survival of pathogens are known inoculum can be reduced by nonchemical cultural methods such as modification of the soil moisture levels, regulated applicaton of organic amendments, selective fertilization intercropping with resistant plants, and/or biological control

The overall aim of this project was to detemine the limits of survival of the soil-borne pathogens (Xcc) in acidic tropical soils and to determine the conditions under which these levels of inoculum will perpetuate disease Early in our collaborative studies we discovered that contaminated seed was a major source of inoculum in Nepal, and infected seed served to reinfest soils even after extended crop rotations We thus undertook studies to measure the levels of seed infestation and to quantify the rate of symptomless spread of disease in seedbeds during the first six weeks of the cropping cycle

B Specific objectives

1 Develop methodology for detecting Xcc and identifying specific strains of this bacterial pathogen using highly specific monoclonal antibodies and a genetically engineered bioluminescent "lux" reporter strain to detect live cells of the pathogen

2 Determine the survival and spread of Xcc strains under different moisture regimes, temperatures, and nutrient levels

3 Devise management practices that will minimize the incidence of black rot disease in Nepal and Hawaii

C Relation to current research At the time this project was proposed, little information was available on the survival of Xcc in soil or its spread on leaf surfaces of crucifers and rotational crops The most common mode of penetration is through the hydathodes (water pores) at leaf margins (Sutton and Williams, 1970), although the pathogen also gains entry through the root system (Cook et al 1952) The pathogen was known to survive in soil for more than a year in undecomposed plant debris in soil (Schaad and White, 1974), but whether or not this amount of inoculum was sufficient to reinfect plants through wounds in roots had not been studied The pathogen is commonly transmitted through seed but infected seed often escapes detection and is planted along with healthy seed Production of crucifer seed in cool areas of temperate zones with low summer rainfall helps to reduce contamination of seed In humid or tropical areas, it is extremely difficult to produce disease-free seed because of the heavy rains that spread bacteria onto flowering parts and seed may be contaminated eventhough the foliage shows no symptoms (Williams 1980) In addition, disease is perpetuated by bacteria that survive in infected leaves and plant debris and for short periods in the soil (Alvarez and Cho, 1978, Williams, 1980) During the course of this project, much new information has been obtained about the specific factors that influence survival and spread of the disease (Schaad and Alvarez 1993 Shaw and Kado 1986 Dane and Shaw, 1996) in addition to the specific information gained through the research in this project (see list of references)

D Innovative aspects of the project Use of hybridoma technology and MAbs for detection and identification of plant pathogenic bacteria provided a novel approach to studies of soil-borne inoculum at the time this project was proposed Previously survival and epidemiological studies were carried out by indirect assay methods using semi-selective media to estimate viable populations of bacteria Indirect estimates were subjected to gross errors because they were based on populations extracted from soil and enumerated under highly artificial conditions Serological methods for detection of Xcc enabled direct observations of root surfaces *in situ*, but these early attempts introduced errors due to the non-specificity of polyclonal antisera (Alvarez and Lou, 1985) Our contribution was to generate highly specific MAbs that identify Xcc and delineate different strains and groups of strains in the general pathogen population (Alvarez et al , 1985

1994) Cross-reactions with unrelated populations of other soil microorganisms were largely eliminated Strains of Xcc collected from nearly all crucifer-growing areas of Nepal were collected and characterized with MAbs, biochemical tests, and pathogenicity assays (Alvarez, et al 1994, Shakya et al *manuscript in preparation*) We found that all the strains from Nepal reacted with at least one or more of the MAbs in the panel, Thus, we were able to assign serotypes to all the strains in the Nepali collection and we were able to use selected antibodies in survival studies on leaf surfaces and soil

Direct serological methods, such as the enzyme-linked immunosorbent assay (ELISA) and immunofluorescence (IF) are unable to distinguish live from dead cells, and this was a potential pitfall forseen in our methodology To resolve this problem we made further modifications in methodology The first modification employed a miniplate-enrichment ELISA that not only excluded dead cells from detection but also enhanced the sensitivity over direct ELISA by 10 000-fold The second modification used the same principle, but employed an immunofluorescence colony-staining technique (Alvarez, et al , 1996) Again, only live cells were detected, and sensitivity was 10,000 times greater than direct immunofluorescence For the third modification we departed from serology and bioengineered a virulent strain of Xcc to contain the "lux" operon, originally obtained from a marine bacterium, *Vibrio fischeri* (Shaw and Kado, 1986) We were unable to use the strain from Shaw and Kado for our studies because the strain they selected was not pathogenic on our model test crop, cabbage We used the plasmid in *Escherischia coli* to transform a virulent strain isolated in Hawaii and then developed a rapid assay using this the transformant as a bioluminescent reporter strain Data were quantified by adapting the most probable number (MPN) method for assays of soil and leaf surfaces (Arias et al , 1996) This method was equal in sensitivity to the miniplate enrichment assay (Norman and Alvarez, 1994) and also distinguished between live and dead Xcc cells on plant surfaces and in soil Once the methodology was in place, we used well-established methods of soil and microbial population analysis to complete the project

E <u>Other organizations that supported the project</u> The Central Department of Botany of Tribhuvan University supported the project by providing facilities, students, and the salary of our chief collaborator, Dr D D Shakya, in Nepal The University of Hawaii

provided laboratories, greenhouse facilities and field plots for the aspects of this study that could not be undertaken in Nepal The U S Department of Agriculture provided supplemental grant funds through the T-STAR program that enabled us to extend the survival studies to the phylloplane We obtained new epidemiological data that provided important insights on symptomless spread of the pathogen in seedbeds and in field plots

III METHODS AND RESULTS

A Isolation and characterization of Xcc strains from Hawaii and Nepal

Methods Crucifer production areas were surveyed for disease incidence and severity Most farms showed high incidence of black rot disease when temperatures were above 28 C (Fig 1) Bacterial strains were isolated from infected leaf materials, seed and soil from the cabbage production areas of Hawaii and Nepal Strains were tested for pathogenicity and reactions with specific monoclonal antibodies, and serological reactivity patterns were compared with strains from a world-wide collection In Nepal bacterial strains were isolated from many different provinces from ranging from the Themi vegetable production region near Kathmandu to the humid, low elevation Terai region and the cooler mountainous seed-production areas (Fig 2) Pathogen identity was confirmed with standard biochemical and pathogenicity tests and serological reaction patterns were compared with Xcc strains from Hawaii that represented the major serogroups

Results Strains from Nepal all had the major biochemical characteristics of Xcc strains in the world-wide collection Characteristics of strains are shown in Table 1 Strains were serologically heterogeneous but all strains fell into the major serogroups represented by the strains from Hawaii and other geographical regions of the world No new serotypes were detected among the 43 strains characterized from Nepal Xcc was commonly detected in seed produced in Nepal, and most of the seed samples collected from the major Asan Market in Kathmandu were infected

Publications Three publications resulted from this initial work and survey on disease incidence in Nepal 1) Alvarez et al , 1994, 2) Shakya, et al , 1998, and 3) Manandhar, U 1994 (See Section VIII)

B Selective media and serological methods for detection of Xcc in soil, on leaf tissues and in seed

Methods We first attempted to utilize selective media as a basis for evaluating serological methods of detection Selective media were evaluated and compared for recovery of Xcc from seed and soil These were then used to develop a enrichment ELISA technique whereby the sample containing bacteria was introduced into individual wells of 96-well microplates and incubated for 72 hours Then the top layer of agar was washed with carbonate buffer removed and dried onto a new 96-well microtiter plate ELISA was performed using selected monoclonal antibodies

Results The method referred to as miniplate-enrichment ELISA, was accurate and 10,000 times more sensitive than direct ELISA for detecting Xcc in soil and on leaf samples Recovery of Xcc by the miniplate-enrichment ELISA method was equal to recovery on two selective media (ET and FS) when soils were dry, but it was 10 to 100-fold more sensitive than either of these selective media when samples were taken from moist or wet soils This is because in wet soils plates were overgrown with other bacteria and it was impossible to identify Xcc when mixed with high levels of background growth

Publications One publication and one abstract resulted from these studies 1) Fukui et al , 1994, 2) Mochizuki and Alvarez, 1992 (See Section VIII)

C Development of a bioluminescent reporter strain for survival studies

Methods Several strains representative of the typical Xcc population in Hawaii were selected for these studies The "lux" gene cassete was introduced to Xcc by triparental matings with E coli strain HB101 (pUCD607) and helper strain The transconjugants were tested for growth in vitro, for virulence following serial passage through plants and were serotyped using the MAbs A stable strain was selected in which the genes conferring bioluminescence were on the chromosome To use this strain in survival studies a method was developed to detect the pathogen in low numbers from seed samples leaf surfaces and soil Samples to be tested are first incubated in individual wells of 96-well microtiter plates containing 100 ul of a semiselective medium, described above A sensitive X-ray film is placed over the microtiter plate to detect the signal from the reporter strain The number of Xcc cells in the original sample is then calculated using a modified most-probable-number (MPN) technique described by Woomer et al 1990)

Results A stable transconjugant, 171LIIH-7, was developed and characterized This strain has been stable throughout the six year duration of the project and has been useful for survival and epidemiological studies

Publications McElhaney et al 1992, 1997 Arias et al 1996a b Arias 1998 (See Section VIII)

D Survival of Xcc in soil

Methods Soil survival studies were carried out on a medial isothermic Typic Eutrandept soil representative of the areas where most of the crucifers are produced in Hawaii and similar to soils in Nepal where crucifers are grown A water retention curve was prepared using ceramic pressure plates by applying -10, -30, -50, -100, -250, -400, -600, and -900 kPa In early experiments, bacteria did not survive more than seven days as free-living organisms in moist

soil Thus, our experiments tested survival in naturally infected cabbage, ground to pass a 1 mm^2 sieve and vacuum-dried overnight Debris was added to the soil at various ratios and the populations of Xcc were evaluated with selective media (Fukui et al 1994) the miniplate enrichment ELISA technique and the MPN-microfluor plate technique (Arias, et al , 1996a) Populations of other microorganisms associated with the decline of Xcc populations was evaluated on selective media (Arias, 1998)

Results Populations of Xcc in soil infested with crucifer debris declined rapidly when soil was moist (low soil matric potential) but showed little decline when soils were dry Populations of cellulolytic microorganisms rapidly increased when water was added to soils but populations of proteolytic organisms stayed relatively constant and were unrelated to the decline in Xcc populations In saturated (wet) soil Xcc declined rapidly and was detected for only 10 days, while in dry soil (-900 kPa) the population remained stable for more than 28 days

Publications Fukui et al 1994 Arias et al 1996a, Arias 1998 (See Section VIII)

E Survival on leaf surfaces of alternate hosts

Methods Epiphytic survival of Xcc was determined on 3-week-old seedlings of cabbage, mustard, lettuce and rice Seedlings were sprayed with a suspension of bacteria containing approximately 10^8 colony-forming units (CFU) per ml) Leaf samples were taken at regular intervals from 1 to 40 days Four basal leaves, which were present at the time of inoculation, were collected and processed from each of four individual plants per species on every sampling date Entire leaves were placed in sterile 250 ml Erlenmeyer flasks containing 20 ml of phosphate buffered saline Flasks were placed on a rotary shaker for 15 minutes then 10-fold dilutions were plated on microfluorplates and processed as indicated for soil samples (Arias et al 1996a) Data were

analyzed with one-way analysis of variance and Scheffe's test for comparison of means of the transformed data were performed within each sampling date using the computer program STATISTIX (Siegel 1992)

Results The pathogen Xcc was recovered for up to 48 days on spray-inoculated cabbage, mustard and lettuce leaves, with no significant differences among these crops (P = 0 05) Declines were gradual after 48 days In contrast, the pathogen was detected on rice leaves for nine days but eleven days after spray inoculation was undetectable Results from this study and the one above indicate that rice is an ideal rotational crop for crucifers The wet soils required for rice production caused rapid declines in Xcc populations, and leaves were not conducive to epiphytic survival Thus, for both reasons, a rotational crop of rice would decrease the survival of Xcc

Publications Arias, et al , 1996b, Arias, 1998 (See Section VIII)

F Spread of Xcc in the seedbed

Methods Seedbeds were established in 8 X 12 arrays to conform with a microtiter plate Four plants at the center of each seedbed were inoculated with Xcc and the flat was watered by an overhead misting system similar to those used in commercial seedbeds Plants were sampled at regular intervals by methods described above Symptomless spread of the pathogen was detected by the miniplate enrichment ELISA technique (Mochizuki and Alvarez, 1992 Norman and Alvarez 1994, Shigaki et al 1994, Shigaki 1996) or in later experiments by the MPN-ficrofluor technique (Arias et al , 1996a, 1996b)

Results In all experiments the pathogen was detectable on symptomless leaves throughout the seedbed and was not restricted to inoculated plants Most plants failed to develop symptoms even after six weeks the normal time for transplant into field plots In one large experiment symptomless plants from

inoculated flats were transplanted into field plots (six treatments, four replications) and disease was monitored to the time of harvest The field had not previously been cropped to any crucifers, and cruciferous weeds collected from the plot showed no signs of disease nor was the pathogen detected by ELISA from any symptomless cruciferous weeds Three weeks after transplanting symptomless crucifers into the field, symptoms began to appear on the transplanted crop Seven weeks after transplant, the entire plot was plot showed no infected, and no plants survived to harvest These experiments demonstrated that a small amount of inoculum can infect an entire seedbed, and because the grower will not be able to recognize and eliminate infected plants, disease can destroy the entire crop This is particularly devastating to small growers, as they will have invested time and resources into getting the crop to maturity

Publications Mochizuki and Alvarez 1992 Shigaki and Alvarez, 1993, Shigaki, et al , 1994 Shigaki 1996 Arias et al 1996ab (See Section VIII)

G Effects of nutrition on disease development in the seedbed

Methods The effects of nitrogen phosphorus calcium, and potassium on cabbage growth and susceptibility to infection by Xcc were determined using a common cabbage cultivar from Hawaii (*Brassica oleracea* var *capitata* cv CG-Cross) Nutrients were added as a balanced Hoagland's solution or as Osmocote formulations Aqueous solutions of ammonium nitrate, ammonium sulhate or potassium nitrate were used to evaluate the effect of various forms of nitrogen on susceptibility to disease The symptomless spread of the pathogen within seedlings were evaluated by the leaf blot assay (McElhaney 1991) using a bioluminescent reporter strain of Xcc Plants were continuously monitored for six weeks for internal presence of the pathogen

Results Among all the nutrients tested, only nitrogen had an effect on the susceptibility of cabbage to black rot Disease progression was similar in all

plants with low and normal levels of nitrogen fertilization, but at 2 X and 3X the normal levels ammonium nitrate and ammonium sulphate disease restricted disease progress Fertilization with potassium nitrate showed similar results, except that while symptoms were clearly reduced, the leaf blots showed that the pathogen had invaded the vasular tissue and progressed well into the main stem These results can be used to modify fertilization practices in the seedbed, where fertilization with higher levels of nitrogen would accomplish two functions 1) restrict disease progress by Xcc, and 2) produce sturdier seedlings prior to transplant Application of more nitrogen to the seedbed would be cost-effective and should provide protection during the seedling stage when epiphytic spread of the pathogen has its greatest impact

Publications McElhaney et al 1998 (See Section VIII)

H Use pesticides and/or biocontrol agents to retard spread of Xcc in the seedbed

Methods The bioluminescent reporter strain of Xcc was used in these studies to determine the effects of single or multiple pesticide applications on disease development in the seedbed Fosetyl-Al was selected for final studies, because this fungicide had bacteriocidal effects Eight replicates of single seedlings were treated with 1, 2 or 3 applications of fosetyl-Al at weekly intervals prior to inoculation of the pathogen Control treatments substituted deionized water for the fungicide suspension Seedlings were inoculated 2 days after the final fosetyl-Al application After 13 days all inoculated leaves were excised and autophotographed using sensitive x-ray film to detect bioluminescence from the reporter strain of Xcc

Results A single treatment of fosetyl-Al as a spray and drench reduced the invasiveness of Xcc on inoculated cabbage seedlings Multiple treatments did not reduce the invasiveness further The effect of fosetyl-Al was immediate (within one hour) and persisted for at least 21 days No phytotoxic effects

were evident following a single treatment The results show that growers can significantly reduce pathogen spread in the seedbed by a single application of a fungicide already registered for use on other vegetable crops

Publications Mochizuki and Alvarez 1996

IV IMPACT AND RELEVANCE OF TECHNOLOGY TRANSFER

A How will the findings be useful in the developing country? The research findings clearly demonstrated that vegetable (crucifer) production is seriously hindered by black rot disease, but that with proper crop management and education losses due to this disease can be prevented (Please refer to extension bulletin) Some of the disease control practices have been well-documented and can be applied directly if extension workers work with growers to educate and assist in implementing control practices

Survival of the pathogen *X campestris* pv *campestris*, in crucifer seed was not a primary objective of the current project since our work was focused on finding methods to reduce survival of the pathogen in soil Nevertheless, findings from our initial surveys in Nepal made it clear that contaminated seed is a major source of inoculum in most production areas Further improvements in crucifer production will require enhancement of seed detection technology, and may require some additional research to adapt state of the art techniques that should be implemented with the facilities available in Nepal (please refer to section VII, Future work) A clean seed program could be developed and adapted to the specific cultural conditions prevelant in vegetable-growing areas of Nepal (both Themi river valley near Kathmandu and the Terai vegetable growing regions)

B The project's impact on individuals, laboratories, departments, and institutions The scientist impacted most directly in this project was our collaborator Dr D D Shakya She received extensive training in use and application of monoclonal antibodies for detection and epidemiological studies and now should be able to direct her students and colleagues in both disease managment practices as well future research on this and other seed-borne vegetable diseases caused by bacteria With the resources provided by this grant, whe was able to supplement the research for two M Sc students at Tribhuvan

University and to extend the information to growers If she is provided the resources to continue a research and extension program at Tribhuvan University, the program will have a continuing impact on her colleagues and the and the outreach programs of Tribhuvan University

C Use of results This project leads to the need for development of a pathogen-free "clean" seed program to be established by the growers and or Ministry of Agriculture in Nepal Such a program could be implemented and supported bv other international organizations, such as FAO or UNDP During our visit to Nepal in 1997 we discussed the potential for a clean-seed program in Nepal with Ms Sandra Vogelsang, U S Ambassador to Nepal Ms Caroll Long Residential Representative of the UNDP to Nepal, and Dr Richard Wuster the Residential Program Officer of FAO in Nepal In addition we discussed potential improvements for Nepal with Fred Pollock, project officer for USAID in Nepal and with Bharat Prasad Upadhyay, the Chief Plant Protection Officer in Nepal

D Are larger scale trials warranted? Yes larger scale trials are warranted provided that resources are available Field demonstration plots could be established in the Themi area (major vegetable production for Kathmandu) to show that with the use of pathogen-free seed plus appropriate cropping practices, removal of diseased debris, proper composting and crop rotations black rot disease can be eliminated from this area and crucifer production will be improved

For long-term improvements, however, Nepal will need to produce its own clean seed in cool, dry high elevation valleys The seed production areas will need inspection followed by seed testing and certification Seed testing methods already used by major international seed companies could be employed by Nepali scientists with adaption to the local situation

E Benefits to developing country Nepali scientists now have the means and methodologies to reduce disease incidence on crucifer crops in the Kathmandu valley (Themi) as well as the Terai regions where the bulk of crucifers are grown and rotated with rice The information is available and the scientists are aware of the major problems the inoculum sources, the survival of inoculum in debris, and the means to eradicate the

pathogen from soil The scientists themselves will not be able to effect the necessary changes without support and cooperation with the Ministry of Agriculture and a well-defined extension program An essential part of the program would be to establish a crucifer-free period of at least three months in any region where crucifers are rotated with vegetable crops of other plant families The program should include the eradication of cruciferous weeds during the crucifer-free period

A second important benefit to Nepal was the identification of contaminated seed sources The scientists now are clearly aware of the need for establishing production areas for pathogen-free seed in Nepal If isolated production areas are established, inspected, and certified, seed produced from such areas would have commercial value not only in Nepal but also in India, which because of climate, will have greater difficulty in establishing production areas for healthy crucifer seed

F How have scientific capabilities of country scientists been improved?

Nepali scientists have gained technical training necessary to implement changes in Nepal They gained experience with serological detection techniques (using ELISA and immunofluorescence) They are now familiar with quantitative approaches to and analysis of research results They also have experience in the use of bioluminescent reporter strains for research Restrictions on use of genetically engineered bacteria may prevent research with reporter strains in Nepal, but the Nepali scientists will be able to continue this research in connection with U S institutions where permits have already been granted Knowledge and awareness of the potential and application of such methods are important to further advances

V PROJECT ACTIVITIES, OUTPUT

A Meetings attended National meetings, American Phytopathological Society (APS) 1992, 1993, 1994, 1996, 1997 1998

B Workshops presented May 15 - 28 1997 Tribhuvan University, Kitipur Nepal

C Training programs for Nepali collaborator and students

D D Shakya, the collaborating scientist from Central Department of Botany, Tribhuvan University, Kitipur, Nepal participated in three training sessions at the University of Hawaii 1) the first was for four months, 1992-1993 2) the second was for one month 1996-1997 3) the third was for one month, 1998 She learned serological techniques with monoclonal antibodies and used them to characterize strains of *Xanthomonas campestris* pv *campestris* isolated from Nepal (*manuscript in preparation*)

Ganga Malla, a M Sc graduate from Tribhuvan University participated in a three-month training session at the University of Hawaii She learned serological techniques using monoclonal antibodies and also learned to study epiphytic populations of *X campestris* pv *campestris* using a bioluminescent "reporter strain developed by R McElhaney in our laboratory

Uttam Manandhar, a M Sc student from Tribhuvan University participated in the project in Nepal, completing field studies under the supervision of D Shakya He also participated in field trips and workshops when we visited Nepal in 1997

Indu Baniya, a M Sc student from Tribhuvan University participated in laboratory studies in Nepal and attended the workshop presented in 1997

D Training and research by students and scientists from Hawaii and other countries

Rosemarie McElhaney, a Ph D student from Hawaii developed a bioluminescent reporter strain and used it to study the effects of nutrition on disease development

Glenn Mochizuki, a M Sc student from Hawaii participated in epidemiology and disease control studies using serology and the bioluminescent reporter strain

Ryo Fukui, a post-doctoral researcher performed initial studies to determine which semiselective media were most adequate for detection of the pathogen in soil survival studies

Toshiro Shigaki, a Ph D student from Japan participated in epidemiological and genetic studies using serologically marked strains to differentiate separate populations of the pathogen

Renee Arias, a Ph D student from Argentina participated in epidemiological and soil survival studies using both serological and genetic markers for pathogen detection

Robustiano Alvarez, Ph D directed the research on detection of the pathogen and survival in tropical soils

VI PROJECT PRODUCTIVITY

We accomplished all of our specific goals, but it took longer to accomplish them than originally intended A major problem was difficulty in communicating efficiently with the collaborator because of power outages and failure of fax machines We did not have

easy access to e-mail with our collaborator, because the only line for e-mail was far from her working site We intended to do more work on field plots in Nepal to test the effects of soil type on the survival of the pathogen However findings from our first experiments clearly indicated that water content and composting were over-riding factors in eradiction of the pathogen from soil Thus we placed more effort on the epidemiological aspects, such as symptomless spread of the pathogen in seed beds Our results showed that a few infected seeds in the seed bed will lead to a major disease outbreak in production fields As a result, we turned our efforts to preparing our collaborators in serological detection methods, so that a clean-seed program can be established in Nepal for vegetable growers

VII FUTURE WORK

A Policy development and extension efforts

Results of this project demonstrated that disease management practices can be implemented to control black rot disease in Nepal (for summary please see extension bulletin) In Nepal disease is clearly spread by infected seed readily available in the major markets Disease management programs for Nepal should focus on two approaches

1) Developing disease-free seed-production areas in high-elevation, cool dry isolated mountain valleys The purchase of certified hybrid seed from international seed companies is also an option, but the high cost of hybrid seed and lack of availability to growers in Nepal is a major concern
2) Coordinating a three-month crucifer-free period in all major vegetable-producing areas whereby growers rotate crucifer crops with non-cruciferous vegetables and preferably, rice Debris from crucifer crops should be composted and used to grow the non-cruciferous crops Rotations with rice have the added advantage of rapid kill of *X campestris* pv *campestris* in the wet soil environment Strict six-week to three month crucifer-free periods would do much to reduce the high level of disease incidence in both the Themi and Terai regions of Nepal

Future research

In Nepal applied research is needed to adapt the newer serological methods of detection of the pathogen in crucifer seed and to use this technology to clearly demonstrate to Nepali growers that both the seed and cruciferous weed crops in Nepal are the major sources of inoculum

In Hawaii future research has been directed toward biological control in the seedbed It was clearly demonstrated that by applying chemical sprays (fosetyl-Al) to the seedbed that spread of symptomless, latent infections was significantly reduced Similar results can now be obtained with a selected biological control agent (Arias

unpublished data) Biological, rather than chemical control of disease in the seedbed has great potential for improving crop production in tropical areas where crucifer crops are transplanted rather than seeded directly in the fields

VIII LITERATURE CITED

A Publications resulting entirely or in part from the current research project

Alvarez, A M , Alvarez R and Shakya, D D 1997 Black rot of cabbage and other crucifers Disease assessment and prevention in Nepal Extension publication/Bulletin (*enclosed*)

Alvarez, A M , Alvarez, R and Shakya D D 1998 Disease assessment and control of black rot of crucifers in tropical and semitropical areas Extension bulletin University of Hawaii College of Tropical Agriculture and Human Resources (*in preparation*)

Alvarez, A M , Benedict A A Mizumoto C Y Hunter J E , and Gabriel, D W 1994 Serological, pathological and genetic diversity among strains of *Xanthomonas campestris* pv *campestris* infecting crucifers Phytopathology 84 1449-1457

Arias, R S 1998 Survival and biological control of *Xanthomonas campestris* pv *campestris* on cabbage (*Brassica oleracea*) Ph D dissertation, University of Hawaii, Honolulu, HI

Arias, R S , Mochizuki R Fukui R and Alvarez A M 1996a Most probable number method to enumerate a bioluminescent *Xanthomonas campestris* pv *campestris* in soil Soil Biol Biochem 28 1725-1728

Arias, R S Nelson S C , and Alvarez, A M 1996b MPN-microfluorplatetechnique to study epiphytic populations of a bioluminescent *Xanthomonas campestris* pv *campestris* Pytopathology 86 S35 (*Abstr*)

Fukui, R , Arias, R S and Alvarez, R 1994 Efficacy of four semi-selective media for recovery of *Xanthomonas campestris* pv *campestris* from tropical soils Journal of Applied Bacteriology 77 534-540

McElhaney, R Alvarez A M and Kado C I 1992 Use of a bioluminescent transconjugant of *Xanthomonas campestris* pv *campestris* to monitor disease progress in cabbage seedlings under different nutritional regimes Phytopathology 82 1177 (*Abstr*)

McElhaney, R , Alvarez, A M and Kado, C I 1997 Nitrogen limits *Xanthomonas campestris* pv *campestris* invasion of the host xylem Physiol and Molec Plant Pathology 52 15-24

Manandhar, U 1994 Incidence of black rot of crucifers and local cultural practices at Manohara-Thimi M Sc Thesis Tribhuvan University, Central Department of Botany, Kitipur Kathmandu Nepal

Mochizuki, G T and Alvarez A M 1992 Immunodetection of *Xanthomonas campestris* pv *campestris* in the guttation fluid of latently infected cabbage seedlings Phytopathology 82 1154 (*Abstr*)

Mochizuki, G T and Alvarez A M 1996 A bioluminescent *Xanthomonas campestris* pv *campestris* to monitor black rot infections in cabbage seedlings treated with fosetyl-Al Plant Disease 80 758-762

Schaad N W and Alvarez A M 1993 *Xanthomonas campestris* pv *campestris* cause of black rot of crucifers Pages 51-55 in Xanthomonas J G Swings and E L Civerolo (eds) Chapman and Hall, New York

Shakya, D D , Manandhar U Malla G Alvarez, R and Alvarez, A M 1998 Serological and biochemical characterization of *Xanthomonas campestris* pv *campestris* strains isolated from crucifer seeds and plants in Nepal *Manuscript in preparation*

Shigaki, T 1996 Differential epidemiological fitness among strains of *Xanthomonas campestris* pv *campestris* and the genetics of pathogenicity Ph D Dissertation University of Hawaii Honolulu, HI, 86 pp

Shigaki, T and Alvarez, A M 1993 Differential epidemiological fitness observed for two strains of *Xanthomonas campestris* pv *campestris* Phytopathology 83 1405 (*Abstr*)

Shigaki, T , Nelson, S C and Alvarez, A M 1994 Greater epidemiological fitness of xcc blight strains in the seedbed Phytopathology 84 1112 (*Abstr*)

B Other literature cited

Alvarez, A M and Cho, J J 1978 Black rot of crucifers in Hawaii inoculum source and disease spread Phytopathology 68 1456-1459

Alvarez, A M and Lou K 1985 Rapid identification of *Xanthomonas campestris* pv *campestris* by ELISA Plant Disease 69 1082-1086

Alvarez, A M , Benedict, A A and Mizumoto, C Y 1985 Identification of *Xanthomonas* and groupings of *Xanthomonas campestris* pv *campestris* strains with monoclonal antibodies Phytopathology 81 857-865

Alvarez, A M , Rehman, F -U and Leach, J E 1996 Comparison of serological and molecular methods for detection of *Xanthomonas oryzae* pv *oryzae* in rice seed Pages 175-183 *in* Seed Health Testing Progress Towards the 21 Century J D Hutchins and J C Reeves (eds) CAB International Wallingford, UK

Cook A A , Walker, J C and Larson R H 1952 Studies on the disease cycle of black rot of crucifers Phytopathology 42 162-167

McElhaney, R 1991 Bacterial bioluminescence a tool to study host-pathogen interactions between *Brassica oleracea* and the bacterial phytopathogen, *Xanthomonas campestris* pv *campestris* in black rot of cabbage Ph D dissertation University of Hawaii, Honolulu, HI 102 pp

Norman D J , and Alvarez, A M 1994 Rapid detection of *Xanthomonas campestris* pv *dieffenbachiae* in anthurium plants with a miniplate enrichment/ELISA system Plant Dis 78 954-958

Dane F and Shaw J J 1996 Survival and persistence of bioluminescent *Xanthomonas campestris* pv *campestris* on host and non-host plants in the field environment J Appl Bacteriol 80 73-80

Schaad, N W and White W C 1974 Survival of *Xanthomonas campestris* in soil Phytopathology 64 1518-1520

Shaw, J J and Kado, C I 1986 Development of a Vibrio bioluminescence gene set to monitor phytopathogenic bacteria during the ongoing disease process in a non-disruptive manner Biotechnology 4 560-564

Siegel, J (1992) STASTIX, Version 4 0 Siegel (ed) Analytical Software St Paul, MN

Sutton and Williams, P H 1970 Relation of xylem plugging to black rot lesion development in cabbage Can J Bot 48 391-401

Yuen, G Y , Alvarez, A M , Benedict, A A and Trotter, K J 1987 Use of monoclonal antibodies to monitor the dissemination of *Xanthomonas campestris* pv *campestris* Phytopathology 77 366-370

Williams, P H 1980 Black rot a continuing threat to world crucifers Plant Disease 64 736-742

Woomer, P , Bennet J and Yost R 1990 Overcoming the inflexibility of Most Probable-Number Procedures Agron J 82 349-353

IX APPENDIX I FIGURES AND TABLES

Figure Captions Observations in farmer's fields in Sanothimi and Monohara in Kathmandu valley, Nepal

(Figures are ordered left to right, then down)

1 Cabbage seedlings transplanted into production field using the traditional local cultural practices Transplants were heavily affected by black rot disease

2 Cabbage seedling showing characteristic V-shaped lesions at the leaf margin

3 Older cabbage plants with characteristic V-shaped necrotic lesions on lower wrapper leaves

4 A small plot of cabbage in farmer's field severely damaged by black rot

5 A section of the plot showing severe necrosis and rot on center plants

6 Harvested cabbage heads showing systemic effects of black rot Vascular tissue is invaded (visible as darkened vascular strands) causing eventual rot of the head as the pathogen and secondary pectolytic bacteria further invade the stock

7 Cabbage plants (left) and mustard plants (right), both showing symptoms of black rot

8 Cauliflower leaves showing V-shaped lesions at leaf margins

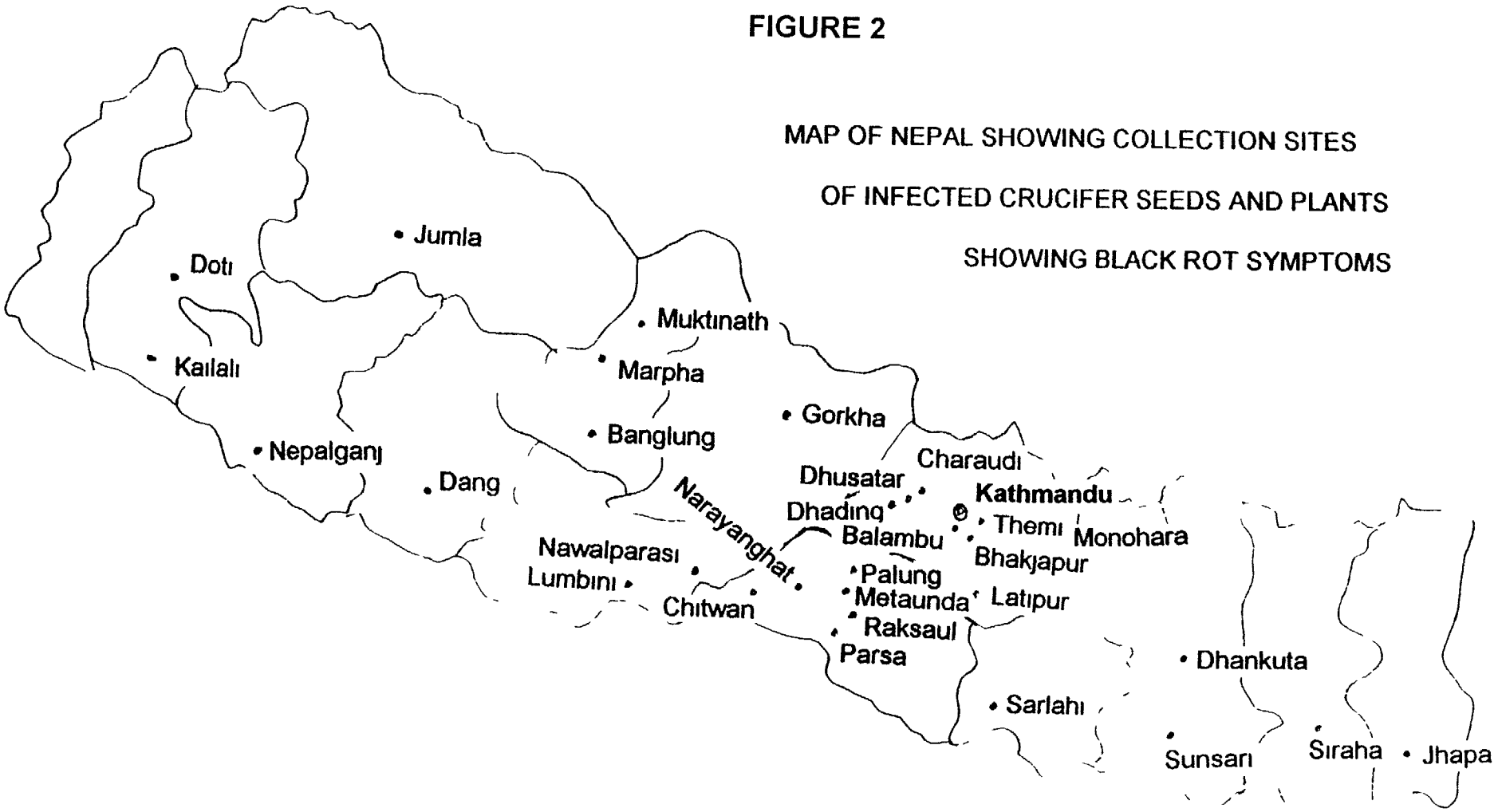
9 A plot of healthy cabbage plants

FIGURE 2

MAP OF NEPAL SHOWING COLLECTION SITES

OF INFECTED CRUCIFER SEEDS AND PLANTS

SHOWING BLACK ROT SYMPTOMS



Xanthomonas campestris pv campestris strains tested with monoclonal antibodies

Strain No	Designation	Origin	Host	Serotype
1	M91-1	Kathmandu Asan Market	mustard	S1
2	M91-2	Kathmandu Asan Market	mustard	S1
3	M91-3	Kathmandu Asan Market	mustard	S1
4	M91-4	Kathmandu Asan Market	mustard	S1
5	M91-5A	Kathmandu Asan Market	mustard	S1
6	M91-5B	Kathmandu Asan Market	mustard	-- [a]
7	C91-1	Kathmandu Asan Market	cabbage	S1
8	C91-2	Kathmandu Asan Market	cabbage	S1
9	C91-3	Kathmandu Asan Market	cabbage	S1
10	C91-4	Kathmandu Asan Market	cabbage	S1
11	GBro-2	Themi	broccoli	S1
12	GRC-1	Themi	red cabbage	S4
13	GRC-3	Themi	red cabbage	S1
14	ThC-1	Themi	cabbage	S1
15	GCa-1	Themi	cabbage	S1
16	GCa-2	Themi	cabbage	S1
17	KGBM-1	Kathmandu	broad leaf mustard	-- [a]
18	PSC-2	Palung	cabbage seedlings	S1
19	PCa-1	Palung	cabbage	S1
20	JCa-3	Jumla	cabbage	S1
21	JCa2-2	Jumla	cabbage	S1
22	JCF-1	Jumla	cabbage	S1
23	BaBM	Balumbo	broad leaf mustard	S3
24	DhC-1	Dhusatar	cabbage	S1
25	DhC-2	Dhusatar	cabbage	S1

26	DhC-3	Dhusatar	cabbage	S1
27	DhC-4	Dhusatar	cabbage	S1
28	ChaC-1	Charaudi	cabbage	-- [b]
29	ChaC-2	Charaudi	cabbage	-- [b]
30	ChiFC-1	Chitwan Fasera	cabbage	S1
31	ChiFC-2	Chitwan Fasera	cabbage	S1
32	ChiKB-1	Chitwan Khairani	broccoli	S1
33	ChiKB-2	Chitwan Khairani	broccoli	S1
34	ChiKK-1	Chitwan Khairani	kohlrabi	S1
35	ChiKK-2	Chitwan Khairani	kohlrabi	S1
36	NapC-1	Nawalparasi	cabbage	S1
37	NapC-2	Nawalparasi	cabbage	S1
38	LuCau-1	Lumbini	cauliflower	S1
39	LuCau-2	Lumbini	cauliflower	S1
40	LuCau-3	Lumbini	cauliflower	S1
41	LuCau-4	Lumbini	cauliflower	S1
42	LuCau-5	Lumbini	cauliflower	S2
43	LuCau-6	Lumbini	cauliflower	S2
44	A249	Kula Hawaii	cabbage	S1
45	Xc114	North Carolina	cabbage	S2
46	G2-17	Kula Hawaii	cabbage	S3
47	G3-27	Kula Hawaii	cabbage	S4
48	417	East Asia	cabbage seed	S4

a) Reacted only with the genus-specific MAbs X1 and X11 weakly virulent
b) Reacted with X1 X11 X17 and B35

X APPENDIX II ATTACHMENTS (PAPERS, ABSTRACTS, and BULLETINS)

Serological, Pathological, and Genetic Diversity Among Strains of *Xanthomonas campestris* Infecting Crucifers

A M Alvarez A A Benedict C Y Mizumoto J E Hunter and D W Gabriel

First author Department of Plant Pathology and second and third authors Department of Microbiology University of Hawaii 96822 fourth author New York State Agricultural Experiment Station Cornell University Geneva 14456 and fifth author Plant Pathology Department University of Florida Gainesville 32611

University of Florida Agricultural Experiment Station Journal Series R-03492 and Hawaii Institute of Tropical Agriculture and Human Resources Journal Series R-03492

This work was supported in part by the U S Department of Agriculture under CSRS Special Grant Agreement 89 34135-4614 managed by the Pacific Basin Administrative Group (PBAG) and the Florida Agricultural Experiment Station

We thank G Lazo R Lipp W Imanaka and G Or for technical assistance

Accepted for publication 9 September 1994

ABSTRACT

Alvarez A M Benedict A A Mizumoto C Y Hunter J E and Gabriel D W 1994 Serological pathological and genetic diversity among strains of *Xanthomonas campestris* infecting crucifers Phytopathology 84 1449 1457

Monoclonal antibodies (MAbs) specific for strains of *Xanthomonas campestris* pv *campestris* (Xcc) and or *X campestris* pv *armoraciae* (Xca) were evaluated to determine their reliability for pathogen identification Reactivity patterns of 1 023 bacterial isolates from crucifer leaves seeds weeds and alternate crops were compared with original host symptoms and reactivity patterns of 498 of these isolates were compared with pathogenicity tests Six MAbs were selected that distinguished strains of Xcc and Xca from nearly all other bacteria tested However Xcc and Xca strains were not serologically distinguished as groups from each other despite numerous attempts to do so Ninety nine percent of the Xcc and Xca strains tested reacted with one or more of the Xcc Xca specific MAbs Three MAbs separated all the Xcc (and some Xca) strains into three serological groups A subset of 12 Xcc and seven Xca strains representing the serological and pathological diversity found in these two pathovars was further evaluated by restriction fragment length polymorphism (RFLP) Strains of both pathovars were heterogeneous by RFLP and the RFLP patterns of the seven Xca strains overlapped with patterns of the Xcc strains However the 12 Xcc strains formed three identifiable groups (C1 C2 and C3) which corresponded to the three serological groups (S1 S2 and S3) The type strain of the species *X campestris* (Xcc528^T) and all members of group S3, C3 caused a distinctive blight symptom that later developed into black rot Strains in the other two Xcc groups caused typical black rot symptoms but not blight The panel of antibodies can be used for rapid identification of Xcc and Xca pathogens in field and seed assays

Additional keywords black vein diagnosis leaf spot

Black rot a serious disease of crucifers that occurs worldwide is characterized by V-shaped lesions on leaf margins and blackened veins associated with systemic movement of the pathogen in the plant (41) The causal organism is *Xanthomonas campestris* pv *campestris* (strains designated Xcc) *Xanthomonas campestris* pv *armoraciae* (strains designated Xca) causes leaf spot and hydathode necrosis on crucifers and on other hosts (6) The genetic diversity of strains within these pathovars has not been well-studied even though pathogenic variation has been noted (21) Strains ideally are placed in different pathovars on the basis of their host range and distinctive disease phenotype (7 10) but in practice strains are often assigned to a pathovar on the basis of the host plant from which they were first isolated (36) Since some pathovars have a host range that involves different families (7) and overlapping host ranges occurrence of a strain of *X campestris* on a particular host should be only one criterion used to classify the pathogen

Several methods have been used with varying degrees of success to differentiate *X campestris* pathovars or subgroups without conducting pathogenicity tests These include DNA-DNA hybridization (19 32) serology (2,5 11 17 38) phage typing (16 26) electrophoretic analysis of membrane proteins (sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE]) (31) gas chromatographic analysis of fatty acids (27) DNA digests (18 24) and restriction fragment length polymorphism (RFLP) analysis (13-15 17 24 25) Of these procedures SDS PAGE and serology have been used in an effort to determine differences among strains of Xcc that cause black rot of crucifers and strains of Xca that cause leaf spot

A high degree of similarity was found among Xcc strains tested by the SDS PAGE procedure (31 38) Serological differences among strains Xcc were detected with monoclonal antibodies (MAbs) which were used to divide 200 strains into six groups (2) Strains that reacted with different MAbs spread at different rates from disease foci in field plots (43) Some evidence of the relatedness of Xcc to Xca was obtained when two strains of Xca reacted with two MAbs that previously had reacted only with Xcc and not with 192 strains of other species or pathovars of *Xanthomonas* (2) Although Xcc was serologically heterogeneous three Xcc Xca-specific MAbs (X9 X13 and X17) reacted with nearly all of 200 strains tested (2) These MAbs thus appeared to be useful for rapid identification of Xcc in seed and plant samples Nevertheless the failure of these three MAbs to detect all pathogenic strains caused concern in the seed industry because it reduces the reliability of the antibody tests Furthermore the reaction of some Xca strains with these MAbs made them less than pathovar-specific

The initial purpose of this study was to evaluate a new and larger panel of MAbs for the ability to identify and distinguish strains of Xcc and Xca from each other and from all other bacteria A total of 521 Xcc and Xca strains isolated from 1 023 plant samples and seed extracts of diverse origins were compared by serology and by symptoms induced on inoculated cabbage plants During the course of the study the serological and pathological diversity observed among strains comprising the two pathovars prompted us to evaluate the genetic diversity of selected strains that represented distinct groups encountered through serological and pathogenicity assays

© 1994 The American Phytopathological Society

27

BEST AVAILABLE COPY

Pergamon

S i B Bi o i V i S N 9p I -I S 1 1 99 El ev S ie I
All ri nt i ved Print d in G eat Britain
0 8 0 7 96 S I 00 + 100

PII S0038 0717(96)00055 7

MOST PROBABLE NUMBER METHOD TO ENUMERATE A BIOLUMINESCENT *XANTHOMONAS CAMPESTRIS* PV *CAMPESTRIS* IN SOIL

R S ARIAS G MOCHIZUKI R FUKUI and A M ALVAREZ
Department of Plant Pathol y U iversity of H iwai HI 968 U S A

(A e ted 6 Januar 1996)

Summary—An adaptation of the most probable number (MPN) method and use of microfluor plates for monitoring a bioluminescent strain (171LIIH 7) of *Xanthomonas campestris* pv *campestris* (Pammel) Dow son in debris inoculated soil is described Microfluor plates with liquid culture medium were inoculated with a dilution series of soil extract previously infested with cabbage debris containing 171LIIH 7 Light produced by the bacteria was detected on X ray film and absorbance readings of the images were converted to a binary set of values for estimation of MPN by the computer program MPNES (Woomer *et al* 1990) The MPN microfluor plate system was compared with direct dilution plating on FS medium and MPN miniplate enrichment ELISA with a pathovar specific monoclonal antibody The detection l mit of X c pv *campestris* with FS medium was about 10^4 cfu g^{-1} soil The detection threshold of MPN microfluor plates (10 bacteria g^{-1} non sterile soil) was equivalent to the MPN miniplate enrichment ELISA and 100 times more sensitive than direct dilution plating The MPN microfluor plate system is an easy accurate sensitive and inexpensive method to detect and enumerate bioluminescent X c pv *campestris* in soil and permits ecological studies in the soil environment that will provide the rationale for changes in crop rotations or other cultural practices
© 1997 Elsevier Science Ltd

INTRODUCTION

Xanthomonas campestris pv *campestris* (X c pv *campestris*) the causal agent of the black rot of cruci ters can survive in host debris and infect plants in subsequent cropping cycles Because of the lack of a sensitive system to trace the bacteria in soil (Schaad and White 1974) the ecology of the soil borne phase of this pathogen has not been clearly established X c pv *campestris* is readily isolated from soil by using various semi selective media (Fukui *et al* 1994) When debris infested soils are moist however the tar get organism is difficult to identify because of over growth by several groups of soil inhabiting bacteria

The development of bioengineered microorganisms to contain a luciferase gene has greatly increased the ability to detect low numbers of marked cells in differ ent environments (Cebolla *et al* 1993) In our system a b olum nescent strain of X c pv *campestris* (171LIIH 7) was developed (R McElhaney unpubl Ph D thesis University of Hawaii 1991) thereby making it possible to monitor populations of this bac terium in soil and from plant surfaces even when nu merous other bacteria including xanthomonads are present

We have adapted the most probable number (MPN) method to microfluor plates for monitoring a biolumi nescent X c pv *campestris* strain in debris infested soil Although a similar approach was taken to enu merate *Pseudom* *e c inosa* in soil using micro ti ter plates (Fleming *et al* 1994) we found that transmission of light among wells limits its use The MPN microfluor plate system we developed overcomes the limitations of the microtiter plates and increases the accuracy and flexibility of the assay

MATERIALS AND METHODS

The bacterial strain we used was X c pv *campestris* 171LIIH 7 This strain is chloramphenicol resistant and has the plasmid pLCD607 containing a *lux* cas sette integrated in the bacterial chromosome (R McElhaney *loc cit*) The *lux* operon within the *lux* cassette operates under the control of the tetracycline resistance gene pLCD607 also confers resistance to ampicil n kanam c n and spectinomycin (Shaw and Kado 1986)

Light detection limits

To test the sensitivity of the MPN Microfluor plate system to monitor small populations of the biolumines cent strain in soil we studied the light detection limits on X ray film in 3 densely pigmented 96 well Microfluor plates (Dynatech Virginia USA) white with flat bottom (W FB) white with U bottom (W UB) and black with U bottom (B UB) and compared them to the microtiter plates used by Fleming *et al* (1994) (Falcon microtest III Becton Dickinson

* Author for correspondence

BEST AVAILABLE COPY

28

Journal of Applied Bacteriology 1994 77 534–540

Efficacy of four semi-selective media for recovery of *Xanthomonas campestris* pv. *campestris* from tropical soils

Ryo Fukui, R Arias and R Alvarez
Department of Plant Pathology University of Hawaii at Manoa Honolulu HI USA

4779/01/94 received 10 January 1994 revised 10 June 1994 and accepted 11 June 1994

R FUKUI R ARIAS AND R ALVAREZ 1994 Four semi-selective media, CS20 ABN, aesculin–trehalose (ET), Fieldhouse–Sasser (FS), and starch–methionine (SM), were compared for efficacy in recovering *Xanthomonas campestris* pv *campestris* from artificially and naturally infested soils Recoveries of *X c campestris* from soils infested with relatively large populations were similar on the four media The FS and ET media exhibited higher selectivity against background saprophytes, whereas enumeration of *X c campestris* on CS20 ABN or SM was often hampered by the overgrowth of background saprophytes Among three starch-containing media (CS20 ABN, ET and FS media), the zones of starch hydrolysis, characteristic of colonies of *X c campestris*, were most distinctive for FS medium This allowed easier identification of the target colonies among numerous non-target colonies in tests with soil containing smaller numbers of *X c campestris* Although the starch zone was also distinctive on CS20 ABN, this medium was not as effective as FS because the starch zones were so large that neighbouring zones fused with each other and many saprophytes formed colonies within the zones Overall, FS was most suitable for soil studies in terms of the consistent recovery of the pathogen, the selectivity against saprophytes, and the differentiation from non-target organisms

INTRODUCTION

Black rot of crucifers, caused by *Xanthomonas campestris* pv *campestris*, is a major disease of cabbage worldwide and threatens cabbage production in Nepal and the Hawaiian islands, particularly in the Kula area on the island of Maui Hawaii Despite the use of pathogen-free certified seeds and other common disease control measures, severe recurrence of disease appears to be perpetuated by the pathogen persisting in soil (Alvarez and Cho 1978) In temperate zones the pathogen survives for 2–6 weeks when free in soil, but may survive for 8 months or longer in infected plant debris in soil (Schaad and White 1974a Graham *et al* 1989) Pathogen populations in soil can be reduced by disking or plowing plant residues to facilitate decomposition of infected debris (Gilbertson *et al* 1990) and some disease control can be achieved by crop rotation and fallowing The population density of soil-borne phytopathogenic bacteria may also be reduced by low winter temperatures (Schuster and Coyne 1974, Saettler *et al* 1986) In Hawaii and Nepal, however, cabbage is produced successively and often simultaneously with other crucifer crops and the mild tropical climate supports the growth of cruciferous weeds (and hence the pathogen) within and around the fields all year around Thus, the disease cycle is continuous Moreover, some phytopathogenic xanthomonads also are known to be capable of surviving in the rhizosphere of the host plant (Goto *et al* 1975) It is likely that the pathogen population persisting in soil and possibly the cabbage rhizosphere has a significant impact on occurrence of the disease

Despite the demand for a practical control method to reduce and maintain the pathogen population below the threshold for disease development, basic ecological and epidemiological studies are constrained by the lack of suitable selective media for detection of the pathogen in soil Although some semi-selective media have been developed, they were inefficient in quantifying the pathogen because of over-growth of background saprophytes (Schaad and White 1974b) or poor recovery efficiency (Kado and Heskett 1970) A starch–methionine medium (Chun and Alvarez 1983) was useful in the isolation of several pathovars of *X campestris* from soil Other selective media, Fieldhouse–

Correspondence to Dr Ryo Fukui Department of Plant Pathology University of Hawaii at Manoa 3190 Maile Way Honolulu HI 96822 USA

BEST AVAILABLE COPY

BEST AVAILABLE COPY

A Bioluminescent *Xanthomonas campestris* pv *campestris* Used to Monitor Black Rot Infections in Cabbage Seedlings Treated with Fosetyl-Al

Glenn T Mochizuki and **Anne M Alvarez**, Department of Plant Pathology University of Hawaii Honolulu Hawaii 96822

ABSTRACT

Mochizuki G T and Alvarez A M 1996 A bioluminescent *Xanthomonas campestris* pv *campestris* used to monitor black rot infections in cabbage seedlings treated with fosetyl Al Plant Dis 80 758 762

Autophotography of a bioluminescent transconjugant of *Xanthomonas campestris* pv *campestris* provided a reliable means to quantify the extent of bacterial invasion into cabbage (*Brassica campestris*) A single prophylactic treatment of fosetyl Al at 4 800 mg kg^{-1} a i as a spray and drench reduced the invasiveness of *Xanthomonas campestris* pv *campestris* on inoculated cabbage seedlings Multiple treatments did not reduce invasiveness further The effect of fosetyl Al was immediate (within 1 h) and persisted for at least 21 days The efficacy of fosetyl Al treatments was more pronounced at 20 C and decreased as temperature increased however the data indicated a separate effect of temperature on disease development and no direct interaction between temperature and fosetyl Al treatment

Black rot caused by *Xanthomonas campestris* pv *campestris* is the most important disease affecting crucifers worldwide (33) Characteristic symptoms include V shaped yellow to tan necrotic marginal foliar lesions followed by vascular darkening stunting wilting and sometimes death of the entire plant Latent infections can occur (1 33)

Foliar infection typically occurs through stomates or hydathodes and infested seeds are the major source of primary inoculum Plants may become infected at any growth stage but the wet crowded conditions in seedbeds are highly conducive to pathogen transmission The pathogen present in the guttation fluid of latently infected but symptomless seedlings can spread to healthy seedlings (21)

Control measures implemented at the seedling stage may significantly reduce the rate of transmission and disease development in the seedbed thereby minimizing the potential for severe field epidemics Very few chemical treatments effectively control bacterial diseases in the seedbed or field Streptomycin oxytetracycline and copper compounds are available but low efficacy pathogen resistance phytotoxicity and visible residues limit their practical use

The systemic fungicide fosetyl Al (Chipco Aliette produced by Rhone Poulenc) reduced the invasiveness of several bacterial diseases of ornamentals when applied as a prophylactic spray (4–7 22 23 32) Weekly fosetyl Al treatments on *Anthurium andraeanum* plants subsequently inoculated with *Xanthomonas campestris* pv *dieffenbachiae* resulted in a significant reduction in disease severity lesion numbers and lesion areas (2 23) Reduction in symptom expression of vascular phytobacterial diseases by fosetyl Al suggested the potential for controlling latent bacterial infections Fosetyl Al is rapidly assimilated into the vascular system and is both acropetally and basipetally translocated resulting in rapid distribution throughout plant tissues (10 11) These properties suggest fosetyl Al as a good candidate for controlling *X campestris* pv *campestris* infections on crucifer seedlings

Recently a genetically transformed bioluminescent *X campestris* pv *campestris* strain containing the *lux* gene cassette was used to monitor infections of cabbage under different nutritional regimes (20) This transconjugant was phenotypically identical to its wild type parent and was constitutively bioluminescent allowing the visualization of infected tissues with autophotography on X ray film This procedure detected the pathogen in presymptomatic tissues suggesting that this method might be useful for evaluating the use of fosetyl Al in reducing latent systemic infection and spread

The objectives of this study were (i) to determine an accurate method of assessing infections with visible and latent components and (ii) to use that method to evaluate the efficacy of fosetyl Al in reducing disease in cabbage seedlings infected with a bioluminescent *X campestris* pv *campestris* transconjugant

Corresponding author A M Alvarez
E mail alvarez@uhunix uhcc hawaii edu

Accepted for publication 9 April 1996

Publication no D 1996 0509 04R
© 1996 The American Phytopathological Society

MATERIALS AND METHODS

The pathogen A bioluminescent transconjugant of *X campestris* pv *campestris* was developed by triparental mating of a highly virulent wild type strain G171 (originally isolated from diseased cabbage in Hawaii) with *Escherichia coli* HB101 containing the *lux* gene cassette in pUCD607 and *E coli* HB101 with the mobilizing plasmid pRK2013 (20) The latter two strains were kindly provided by Shaw and Kado (30) The resulting transconjugant designated strain 171LIIH 7 contained chromosomally integrated *lux* genes and expressed constitutive bioluminescence in culture and in planta (20) This strain was identical to its wild type parent in all aspects studied including pathogenicity virulence growth rate nutritional requirements and serological and physiological characteristics (20)

Seedling preparation and fosetyl Al treatment Cabbage (*Brassica campestris* L) seeds of cv Early Jersey Wakefield were assayed for xanthomonads Seeds were sown into 9 0 cm square plastic pots containing 650 cm^3 of a 2 1 (vol/vol) mixture of Supersoil/vermiculite medium amended with 3 kg of Osmocote 17 6 12 (Grace Sierra Horticultural Products Co Milpitas CA) and 5 kg of dolomite per cubic meter of medium and allowed to germinate on the greenhouse bench Plants were thinned to a single plant per pot and fertilized again with 1 35 g of Osmocote per pot 3 weeks after emergence Plants were watered twice daily except for 24-h periods following fosetyl Al treatment or inoculation Uniform 2 to 3 week old seedlings at the three to five true leaf stage were selected for all experiments

Seedlings were sprayed and drenched with a 4 800 mg kg a i fosetyl Al suspension in deionized water Foliage surfaces were sprayed until runoff (approximately 2 ml per plant) using a hand atomizer then 25 ml of the suspension was drenched directly onto the medium in each pot Watering was withheld for 24 h post-treatment Nontreated control plants were sprayed and drenched with deionized water

Inoculum preparation and inoculation method Inoculum was prepared by suspending cells from a 24 h culture of *X campestris* pv *campestris* strain 171LIIH 7 grown on peptone glucose agar medium (peptone 1% glucose 0 5% agar 1 7%) into sterile 0 01M phosphate buffered saline pH 7 2 and adjusted to a concentration

30

BEST AVAILABLE COPY

Physiological and Molecular Plant Pathology (1998) 52, 15–24

Nitrogen limits *Xanthomonas campestris* pv *campestris* invasion of the host xylem

R McElhaney[1], A M Alvarez*[1] and C I Kado[2]

[1] Department of Plant Pathology, College of Tropical Agriculture, University of Hawaii, 3190 Maile Way, Honolulu, HI 96822, and [2] Department of Plant Pathology, University of California, Davis, CA 95616, USA

Accepted for publication 10 October 1997

Hydathodes are the primary portal of entry for *Xanthomonas campestris* pv *campestris* in cruciferous plants. Once entry is gained, this vascular pathogen quickly multiplies and ramifies progressively in the xylem. It was shown previously that *X campestris* loses its motility when it interacts with the plant host, and its movement in the xylem is relatively passive; nonetheless, the bacterial cells are able to continue to spread in the xylem. The mechanism of bacterial invasion in the xylem has remained largely unidentified. In the present study, *X campestris* pv *campestris* chromosomally tagged with a constitutive *lux* reporter cassette was used for real time analysis of the invasive characteristic of this xylem invading pathogen as a function of host nitrogen nutrition. High levels of nitrogen were found to slow down and even impede the invasive activity of *X campestris* pv *campestris*. The type of nitrogen did not appear to affect the growth rate of *X campestris* pv *campestris*. Each nitrogen type had an appreciable effect on curbing bacterial invasion of the xylem in cabbage plants. These studies suggest that host defence factors other than the conventional hypersensitive response are enhanced by nitrogen in the xylem.

© 1998 Academic Press Limited

INTRODUCTION

Xanthomonas campestris pv *campestris* infects plants through hydathodes (natural openings at the leaf margins) in the absence of wounding, a feature that is common among xanthomonads, allowing the organism to invade and progressively multiply in the xylem elements throughout the foliar parts of cruciferous hosts [2]. Forward movement of *X campestris* pv *campestris* in the xylem is not due to motility or chemotaxis, as the bacterial cells are nonmotile and nonchemotactic in the host plant [10]. In the free-living state, however, they convert to motile, chemotactic forms by an environmentally induced phase switching mechanism [10]. The degree of progression in the xylem by *X campestris* pv *campestris* is dependent on its ability to concomitantly multiply, move in this vascular element, and evade triggering the hypersensitive response of the plant defence system. Although the precise mechanism of host defence evasion is not known for *X campestris* pv *campestris*, it is known that at least the product of a chromosomal gene, *hrpXc*, plays an important regulatory role in conferring this property on *X campestris* pv *campestris* [9, 11]. The *hrpXc* gene is a member of the *araC* gene family [15].

* To whom correspondence should be addressed.

Abbreviations used in text: *araC*, gene C of the arabinose operon; Cm, chloramphenicol; HR, hypersensitive response; Km, kanamycin; *lux*, bacterial luciferase; Sp, spectinomycin.

0885–5765/98/010015+10 $25.00/0 pp97/0151 © 1998 Academic Press Limited

BEST AVAILABLE COPY

DIFFERENTIAL EPIDEMIOLOGICAL FITNESS AMONG STRAINS OF *XANTHOMONAS CAMPESTRIS* PV CAMPESTRIS AND THE GENETICS OF PATHOGENICITY

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BOTANICAL SCIENCES (PLANT PATHOLOGY)

MAY 1996

By

Toshiro Shigaki

Dissertation Committee

Anne M Alvarez, Chairperson
Dean W Gabriel
Suresh S Patil
Scot C Nelson
Sophia Kathariou
John J Cho

32

Abstract

Variation among strains of *Xanthomonas campestris* pv campestris, the causal organism of black rot of crucifers, was studied The relationship between symptoms associated with selected strains, their epidemiological fitness in misted seedbeds, the presence of a gene associated with severe symptoms in these strains, and a pathovar-specific surface antigen was examined Strains with blight induction capacity spread more rapidly in misted seedbeds These blight strains all hybridized with a 5 4-kb DNA fragment isolated from the type strain of *X c* campestris (Xcc528^T) This fragment in pJC41 was previously reported to confer blight symptom induction in cabbage when mobilized to a closely related leaf spot pathogen *Y c* armoraciae The relationship between this 5 4-kb DNA fragment and blight symptom induction was studied by marker-exchange mutagenesis of Xcc528^T at the pJC41 region Loss of the fragment did not destroy the systemicity and blight induction capability of the wild type However, in pathogenicity studies strains of *X c* campestris that hybridized with pJC41 generally caused strong blight in cabbage Thus, it is likely that other gene(s) with similar functions exist on the genome A positive reaction to the monoclonal antibody (MAb) A11 was associated with strains that produced blight The hypothesis that the antigen reactivity with this MAb plays a role in blight induction was tested by mutagenizing a blight strain of *X c* campestris (CAM19) at a gene locus that is necessary for the production of this antigen Although the two A11-negative mutants (NTG901 and NTG2230) which were obtained were avirulent or had reduced virulence, the restoration of antigenicity by complementation did not result in simultaneous restoration of virulence Thus, although the antigen for MAb A11 is a useful

33

marker associated with blight-causing strains, it does not play a role in blight symptom induction or pathogenicity In summary, blight strains of *X c* campestris are distinct from the more typical black rot strains in symptom induction and epidemiological capability They usually, but not always, hybridize with pJC41 and react with MAb A11 It is suspected that blight induction is controlled by multiple genes

MANUSCRIPT IN PREPARATION
ARIAS, 1998

R S Arias S C Nelson and A M Alvarez 1998 The survival of the bacterial pathogen *Xanthomonas campestris* pv *campestris* (*Xc campestris*) was studied in debris infested soil with different water contents (0 10 0 50 100 200 and −900 kPa) and on the phylloplane of crops used for rotation with cabbage Increase in soil water content was negatively correlated with survival of *Xc campestris* and positively correlated with the abundance of cellulolytic microorganisms The time to eliminate a population of 10^6 *Xc campestris* at kPa was 19 to 28 days while at −900 kPa this number remained constant Survival of *Xc campestris* on the phylloplane of cabbage (host) was comparable to mustard and lettuce (48 days) whereas on rice the pathogen was detected for only nine days We conclude that high water contents applied to infested soil and appropriate selection of rotation crops are necessary to reduce the number and spread of *Xc campestris* in the field

INTRODUCTION

Xc campestris the causal agent of the black rot of crucifers is considered a seed borne pathogen (Cook *et al* 1952) Once established in a cropping area the ability of the bacterium to survive in soil within plant tissues and to colonize non host phylloplanes [illegible] count for the recurrent epidemics of black rot disease Nevertheless there are no [illegible] effect of changes in the soil environment to reduce the survival of *Xc campestris* and [illegible] information on the colonization of non host plants by *Xc campestris* This paper addresses the survival of *Xc campestris* in debris infested soil with different water content and on the phylloplane of crops used for rotations with cabbage

Xc campestris can survive in infested plant residues making a significant contribution to the black rot epidemics Infested stems of cabbage (*Brassica campestris* var *capitata*) placed in soil harbored this pathogen for at least six months (Schaad and White [illegible]) [illegible] infested areas

BEST AVAILABLE COPY

Incidence Of Black Rot Of Crucifers And Local Cultural Practices At Manohara-Thimi

By

Uttam Manandhar

A Dissertation Submitted For The Partial Fulfilment Of M.Sc. Degree In Botany

Central Department Of Botany
Tribhuvan University, Kirtipur
Kathmandu, Nepal.

(1994)

36

BEST AVAILABLE COPY

SUMMARY

The incidence of bacterial black rot disease caused by *Xanthomonas campestris* pv *campestris* in different growth stages of cabbage and cauliflower was observed in both summer and winter season at Manchala-Thimi. The characteristic symptom of the black rot disease, the typical black to brown necrotic V-shaped lesion at the margin of the leaves was found in both young and mature plants. The percentage of the infection in younger plants was low. The level of the infection and percentage of the diseased plant was low in winter. Devastation of middle stage or mature cabbage field plot occurred mainly after the few showers of rain in summer.

Altogether 18 cabbage field plots and [illegible] cauliflower field plots were observed. Among these, [illegible] cabbage field plots and 1 cauliflower field plots were disease free. The causal organism Xcc was confirmed by some diagnostic biochemical and pathogenicity test.

Though the farmers of Manohala-Thimi are diligent and skillful in traditional farming practice, they lack consciousness about modern agricultural practices such as seed-treatment and use of pesticides.

It was clearly evident from the present study that the prevalence of black rot disease at Manchala-Thimi is at alarming state. Therefore, it needs an urgent investigation on primary inoculum sources as well as other epidemiological aspects of the disease to under take the timely control measures.

A849

IMMUNODETECTION OF *XANTHOMONAS CAMPESTRIS* PV *CAMPESTRIS* IN THE GUTTATION FLUID OF LATENTLY INFECTED CABBAGE SEEDLINGS G.T. Mochizuki and A M Alvarez Dept of Plant Pathology University of Hawaii Honolulu HI 96822

The hypothesis that *Xanthomonas campestris* pv *campestris* (*Xcc*) could be recovered from the guttation fluid of latently infected cabbage seedlings was tested using three inoculation methods and assayed using a monoclonal antibody (mAb) based immunofluorescent colony staining technique (IFC) to recover viable cells of the pathogen present in low populations Ten day old cabbage seedlings were inoculated with *Xcc* by applying 1 to 10 cfu directly into guttation droplets on hydathodes by infiltrating 10^4 cfu into the root zone or by vacuum infiltrating the seed and growing out the seedlings Plants were kept in growth chambers at 22C to retard symptom expression Guttation was induced and the droplets were collected at 48 hr intervals and assayed using IFC and standard dilution plating Recovery of *Xcc* was intermittent and varied from 2 to over 2000 cfu per seedling The IFC assay using *Xcc* specific mAb X21 FITC was more sensitive and reproducible than standard plating techniques The minimum times for the appearance of *Xcc* in guttation fluid for hydathode root and seed inoculation methods were 2 9 and 20 days respectively These data suggest the possible role of undetected infection and transmission of *Xcc* in the seedbed

A1088

USE OF A BIOLUMINESCENT TRANSCONJUGANT OF *XANTHOMONAS CAMPESTRIS* PV *CAMPESTRIS* TO MONITOR DISEASE PROGRESS IN CABBAGE SEEDLINGS UNDER DIFFERENT NUTRITIONAL REGIMES R McElhaney A M Alvarez, and C I Kado University of Hawaii Honolulu HI 96822 and University of California Davis, CA 95616

A bioluminescent transconjugant of *Xanthomonas campestris* pv *campestris* (Xcc), 171 LIIH 7 was isolated and compared to the wild type for growth *in vitro* and *in planta* The strain remained stable with respect to bioluminescence and virulence over six serial passages through cabbage seedlings Host nutrition and its relation to black rot of cabbage was studied in *Brassica oleracea* cv *capitata* (C G cross) seedlings using this transconjugant In seedlings grown with different levels of NH_4NO_3 $(NH_4)_2SO_4$ KNO_3 K, and P for four weeks prior to inoculation, insufficient amounts of nitrogen in all forms increased disease whereas NH_4NO_3 and $(NH_4)_2SO_4$ at twice the level required for optimum growth reduced the invasiveness of the pathogen Movement of the bacteria in the vascular system of the leaf was confirmed with X ray film Visual lesions were reduced with high levels of KNO_3, but leaf blots revealed that bacteria had extensively invaded the petiole H gher levels of K and P fertilization had no apparent effect, however, when P was omitted, disease development was greatly inhibited The bioluminescent pathogen proved to be a valuable tool in nutritional studies because visual symptoms did not reflect the extent to which the pathogen had progressed in cabbage leaves

ABLE COPY

327A **MPN-microfluorplate technique to study epiphytic populations of a bioluminescent *Xanthomonas campestris* pv *campestris***

R S Arias S C Nelson and A M Alvarez Department of Plant Pathology University of Hawaii Honolulu HI 96822

Crops rotated with crucifers in Hawaii harbor epiphytic populations of *Xanthomonas campestris* pv *campestris* (*Xcc*) that serve as inoculum when cabbage is planted The survival of *Xcc* on leaf surfaces of alternate crops was studied using a bioluminescent strain (171LIIH 7) of *Xcc* Lettuce cabbage mustard and rice plants were spray-inoculated with 171LIIH 7 and leaves of each crop were sampled at intervals from 25 to 40 days in separate experiments Leaf extracts containing bacteria were placed in flat-bottom microfluorplates containing liquid medium, and wells reflecting light emitted by bacteria were recorded on x-ray film The MPNES computer program (Woomer *et al* 1990) was used to estimate the most probable number (MPN) of epiphytic populations of 171LIIH 7 Survival of 171LIIH 7 was similar on cabbage mustard and lettuce and detectable at 10 cfu/cm^2 of leaf surface after 40 days On rice however *Xcc* was detectable for only 12 days Epiphytic spread of *Xcc* was reduced on cabbage after spraying with bacterial antagonists isolated from cabbage seedlings MPN microfluorplate method is accurate sensitive and inexpensive

BEST AVAILABLE COPY

372

GREATER EPIDEMIOLOGICAL FITNESS OF *XANTHOMONAS CAMPESTRIS* PV *CAMPESTRIS* BLIGHT STRAINS IN THE SEEDBED T Shigaki, S C. Nelson, and A.M Alvarez. Department of Plant Pathology University of Hawaii at Manoa 3190 Maile Way Honolulu, HI 96822.

Previous data suggested that *Xanthomonas campestris* pv *campestris* typical black rot (BR) and blight (BL) strains differ in attributes of epidemiological fitness (Phytopathology 83 1405) To assess this conclusion, additional strains from BR and BL groups were compared for latent spread from introduced disease foci in two runs of an experiment in misted seedbeds Samples from leaf wash collected from each plant were incubated on a selective medium in separate wells of microtiter plates and examined by ELISA with monoclonal antibodies that distinguished strains of the pathogen In run one (avg temp 29 3°C), incidence of BL strains 14 days after inoculation was higher (19 1, 9 8, and 8 7% for strains G2-17, A3263, and G2 12 respectively) than for BR strain (6 0, 3 3, and 1 1% for GAC17, A249, and GAC137) In run two (avg temp 26 4°C), incidence was 14 7, 12 0, 10 9, 14 7, 7 6, and 8 7% (in the same order as in run one) Spatial analysis of the final incidence indicated that BL strains spread farther than BR strains The data suggest greater epidemiological fitness of BL strains

A657

DIFFERENTIAL EPIDEMIOLOGICAL FITNESS OBSERVED FOR TWO STRAINS OF *XANTHOMONAS CAMPESTRIS* PV *CAMPESTRIS* T Shigaki and A M Alvarez Department of Plant Pathology University of Hawaii at Manoa Honolulu HI 96822

Latent spread of *Xanthomonas campestris* pv *campestris* was confirmed in cabbage seedbeds by using pathovar specific monoclonal antibodies (MAbs) Bacteria collected from guttation droplets of symptomless plants were grown on esculin trehalose medium in microtiter plates and identified by ELISA by strain specific MAbs One strain (G2 12) which causes blight like symptoms and reacts with MAb A11 spread more rapidly than another strain (A249) which causes typical black rot symptoms and reacts with MAb X21 Four weeks after inoculation the mean incidence was 19 6% and 4 8% for G2 12 and A249 respectively Of seedlings infected with G2 12 and A249 9 1% and 22 2% respectively showed visual symptoms This differential spread may be related to strain differences in epidemiological fitness or efficiency of infection through hydathodes Rapid latent spread in the seedbed may be a significant source of initial inoculum for field epidemics

A849

IMMUNODETECTION OF *XANTHOMONAS CAMPESTRIS* PV *CAMPESTRIS* IN THE GUTTATION FLUID OF LATENTLY INFECTED CABBAGE SEEDLINGS G.T. Mochizuki and A M Alvarez Dept of Plant Pathology University of Hawaii Honolulu HI 96822

The hypothesis that *Xanthomonas campestris* pv *campestris* (*Xcc*) could be recovered from the guttation fluid of latently infected cabbage seedlings was tested using three inoculation methods and assayed using a monoclonal antibody (mAb) based immunofluorescent colony staining technique (IFC) to recover viable cells of the pathogen present in low populations Ten day old cabbage seedlings were inoculated with *Xcc* by applying 1 to 10 cfu directly into guttation droplets on hydathodes by infiltrating 10^4 cfu into the root zone or by vacuum infiltrating the seed and growing out the seedlings Plants were kept in growth chambers at 22C to retard symptom expression Guttation was induced and the droplets were collected at 48 hr intervals and assayed using IFC and standard dilution plating Recovery of *Xcc* was intermittent and varied from 2 to over 2000 cfu per seedling The IFC assay using *Xcc* specific mAb X21 FITC was more sensitive and reproducible than standard plating techniques The minimum times for the appearance of *Xcc* in guttation fluid for hydathode root and seed inoculation methods were 2 9 and 20 days respectively These data suggest the possible role of undetected infection and transmission of *Xcc* in the seedbed

BEST AVAILABLE COPY

FINAL REPORT OF THE COLLABORATOR

Covering Period 1992 to 1998

SURVIVAL OF PLANT PATHOGENIC BACTERIA IN TROPICAL SOILS TRACED WITH MONOCLONAL ANTIBODIES

Collaborator Dr Dilli Devi Shakya, Professor
Central Department of Botany
Tribhuvan University Kitipur
Kathmandu, Nepal

Project Number 12 691
Grant Number 936 5600 (HRN - 5600-G-00-2009-00)

Report on the work completed in Nepal with the assistance from the University of Hawaii

41

Table of contents

1 Executive Summary

2 Research Objective

2 1 Importance of Black Rot Problem in Nepal

2 2 Main Objectives

2 3 Innovative Aspects of Project

2 4 Support From Other Organization

3 Methods and Results

3 1 Field Survey

3 2 Detection, Isolation and Identification

3 3 ELISA Test

4 International Visits

4 1 Tribhuvan University to Hawaii University

4 2 Hawaii University to Tribhuvan University

5 Impact of Project

1 Executive Summary

In Nepal, crucifer crops (cabbage, cauliflower, mustard, broad leaf mustard, radish, rape, broccoli and turnip) are important vegetables These crops are considered as winter crops Black Rot of crucifers caused by the bacterium *Xanthomonas campestris pv campetris (Xcc)* has been found in all areas of crucifer production So far the black rot disease is considered to be severe in the hot humid regions worldwide However in Nepal the disease has been surprisingly occurring in cool temperate and alpine climate even at altitude of above 2000m Farmers of Nepal are very good in their traditional agricultural practices but lack the knowledge of modern technology basically due to lack of education During the field investigation made in this project farmers of most of the important vegetable growing areas had good opportunity to get the knowledge and become almost aware of this particular problem Otherwise almost all farmers think black rot caused by Xcc is due to weather condition rather than pathogenic cause just as in 1845 when potato growers in Europe atributed late blight of potato to weather rather than to fungal pathogen, *Phytophthora infestans*

The causal bacterium Xcc isolated from different hosts of crucifer seeds and infected plant materials were identified on the basis of pathogenecity, biochemical serological tests The serological similarity of Xcc strains of Nepal and Hawaian strains tested by Enzyme-Linked Immunosorbant Assay (ELISA) has open the door for the quick and reliable identification of Nepalese strains with antibodies generated in UH Future collaborative work could be done to do more extensive work in Nepal which has great seed production potential due to natural bounty and cheap labor cost The bacterium Xcc survived on infected seeds longer when stored at room temperature Infected crucifer debris also infect the next crops of crucifers Infested seeds are the potential innoculum source to spread and perpetuate the dissease

2 Research Objective

2 1 Importance of Black Rot Problem

In Nepal , crucifer crops (cabbage, cauliflower, mustard, broad leaf mustard radish rape broccoli and turnip) are most important vegetables These crops are considered as winter crops Black rot of crucifers caused by the bacterium *Xanthomonas campestris pv campestris (Xcc)* is known to be destructive disease world wide in humid and warm climate(williams, 1980) The pathogen survives on seeds (Schaad, 1989) and in soil (Schaad & White, 1970, Chun & Alvareze, 1983, Seattler et al , 1989) as the primary source of inoculum The bacterium had been detected from seeds of mustard, cauliflower cabbage and turnip collected from warm temperate and sub-tropical parts of Nepal and its characterization had been reported (Shakya & Malla, 1988) However, in Nepal crucifers is the important crops also in cool temperate and Alpine climate above 2000 m altitude Cabbage seeed production are undertaken mostly in these areas Very few studies studies had been done on this important disease in Nepal

2 2 Main objectives

- to know the incidence and severity of black rot in the important crucifer growing areas

- to know the cultural practices followed by the farmers

- to detect and assay crucifer seeds for Xcc collected from different sources

- to identify and characterize Nepalese strains of Xcc

- to know whether the Nepalese strains of Xcc will react with monoclonal antibodies (Mabs) already generated in University of Hawaii (UH) eventually to know the serological groups of Nepalese strains

- to suggest control strategies

- to disseminate the results nationally and internationally

2 3 Innovative aspects of project

- The study has clearly demonstrated that the black rot of crucifers is not only the problem of warm hot humid climate but also is the potential problem of cool temperate and alpine climatic condition

- It helped to make the Nepalese farmers aware of the cause of black rot due to bacteria, otherwise more than 95% of farmers on average think it is not a disease but only as weather effect

- For the first time, the occurrence of black rot of crucifers has been recorded from all 14 development regions of Nepal, indicating the disease is of great significant in the country It is the concern of all personeals working in agricultural fields

- Brochures on Disease assessment prevention and crop managment in Nepal will be of great help to aware the agriculturists and farmers of Nepal

- It is generally considered that incidence of black rot dose not occur in the fields where crops are rotated with rice However, the perpetuation of the disease even in such crop rotated fields which is the general practice in Nepal, indicates that seeds, wild hosts and debrises play important role as the primary source of innoculum potential of Xcc

- The seeds stored in room temperature in temperate climate, Xcc servived for more than a year thus seeds can play as most potential carrier

- The study reports for the first time the serogroups of Nepalese strains of Xcc which are similar to those found in Hawaii and many other geographical regions worldwide suggesting common strategic plans to control Xcc could apply for Nepal

2 4 Support from other organization

- Seeds were provided from various Government and Non-Government organizations like Institute of Agriculture and Animal science Pkhribash Agriculture Farm, National Agriculture Research council, FAO vegetable Project, Jumla Agriculture Extension Station and Marpha Agriculture Extension Station etc

- USAID, Nepal organized and sponsored the seminar talk program for Dr Anne Alvarez and Dr R Alvarez and collaborator Dr D D Shakya during their visit to Nepal in the year 1998 (Itinerary see appendix)

- Curtesy call to Her Excellency US Ambassador to Nepal Mrs Sandra Vogelsang, Dr R Wuster, Regional FAO Program officer

3 Methods and Results

3 1 Field survey

Field surveys were undertaken in small farms and commercial crucifes fields of different parts of Nepal ranging from Tarai, Hills and Mountains, covering all 14 developmental zones Evalution of disease severity was recorded and collection of diseased plant materials were randomly collected from all 3 broad climatic regions Seeds of cabbage, cauliflower and mustard were collected from local markets and agricultural farms

In tarai and hills these plants are generally rotated with rice Some of the places the grow crucifers and rice side by side (But in some of vegetable fields cultivation of crucifers are found in all year round

Incidence of black rot disease had been observed in all three major climatic and topographically diverse regions of Nepal e g Tarai in lowest elevation below 300 to 600 m and sub-tropical climate, Hills elevation 600 to 1500 m temperate climate and Mountains above 1500 with alpine and tundra climate The disease was found more severe in sub-tropical and warm temperate regions However, the disease was also prevalent in some fields at high altitude and cool temperate and alpine climatic condition such as in Daman, Jumla, Marpha and Muktinath The morning and evening dew and fog and strong wind might have spread the disease

Detailed study in incidence of black rot was done at Themi one of the important vegetable farms situated at the middle of Kathmandu valley(Manandhar,1994) The altitude of this area is 1280 m and climate is warm temperate Black rot symptoms were found on seedlings to mature stages of growth

Farmers of Nepal are very good in their traditional agricultural practices They make very good compost manure and cropping pattern and sequence But they lack the knowledge of modern technology Based on the response of the questionnaires they were found very much innocent on application of pesticides and even the chemical fertilizers They were using different pesticides without knowing the proper use of related pesticides or dose At the time of spray none of the farmers were using mask or gloves They even do not hesitate to over dose pesticides According to the farmers of that locality (Themi) they have been using chemical fertilizer only since nearly 45 years The pest problems have been increasing since then Farmers were generally using pesticides like Indophil M-45 Dithane, Metacide, Nuvan, Vepona, Fen Fen 20 and BHC Although BHC had been banned during our survey several farmers were still using it and they were advised not to use it

3 2 Detection, Isolation and Identification

The causal bacterium was isolated (Table) in the laboratories of Tribhuvan University (TU) and University of Hawaii(UH) from infected plants materials and seeds collected from different zones of the country Bacteria from crucifer seeds were extracted following Schadd 1989 utilizing both direct platting and seed extracts on different semiselective media e g CS20ABN (Chang et al 1991), Fieldhouse Sasser medium FS (Yuen and Alvarez, 1987) The isolates were identified on the basis of biochemical, serological and pathogenecity tests(Shakya et al 1993, Shakya and Manandhar 1995)

3 3 ELISA Test (Enzyme -Linked imminosorbant Assay)

ELISA test with monoclonal Antibodies generated in the University of Hawaii against Xcc were used to find the serological groups of Nepalese's Strains of Xcc (Yuen et al , 1987) during my three visits to UH in the years 1992/93, 1996/97 and 1998 Xcc strains isolated from crucifer seeds and plant materials from Nepal had positive reactions with several Monoclonal antibobies (Mabs) generated in the laboratory of UH These serological reactions were typical of many Xcc strains isolated from Hawaii (Table) The serological similarity of the Xcc strains to those of Hawaii is not unexpected since these strains all fall into serologically typical of Xcc strains from a worldwide collection (Alvarez et al 1985) Selected pathovar specific Mabs, such as X1, X3, X4, X9, X11, X21, and B35 will thus be useful for rapid identification of Xcc from crucifer seeds and fields in Nepal These results provide a foundation for field inspection and seed certification in Nepal

4 Travel and visits

4 1 International Visits

4 2 Tribhuvan University to University of Hawaii

The collaborator Dr D D Shakya, Professor of plant pathologist from Central Department of Botany, Tribhuvan University made three visits to laboratory of Department of Plant Pathology, University of Hawaii on following dates and periods

- First visit 1992/93 -4 months
- Second visit 1996, Dec 12 to 1997, Jan 7
- Third visit 1998, June 25 to July 26

During these visits Dr Shakya had good oppertunity to leaarn specially all aspects of the serological technique which was used to identify the Xcc strains isolated from seeds and plant materials from Nepal Further more, with this technique for the first time the sero-group of Xcc strains of Nepal and their comparision with other international strains had been made In collaboration with UH, this technique can be used in Nepal on seed certification and to devise the strategies for the control measures of black rot diseases using the Mabs already generated in UH

Ms Ganga Malla, Lecturer of Tribhuvan University also had an oppertunity to work partially in the laboratory of UH in the year 1994 She was learning a new technology on using Lux-gene to trace Xcc on crucifer plants

4 2 Hawaii University to Tribhuvan University

Principal Investigator Dr Anne Alvarez , Professor of plant pathology, University of Hawaii and Dr R Alvarez, visited Tribhuvan University Nepal in the 1997, May16 to 26 The Programes,

-Visit of Tribhuvan University and discussion with with the collaborator Dr D D Shakya

- Field suveys to observ the black rot incidence and to get acquainted with farmer's cultural practices

- Workshop and Discussion with plant pathologists of Tribhuvan University, Ms G Malla, U Manandhar, I Bania and R Malla

- courtesy call on and briefing of the project

Her Excellency Ambassador of USA to Nepal, Mrs Sandra vogelsang

Residential Reprensative UNDP to Nepal, MS Caroll Long

Resential Program officer FAO, Dr Richard Wuster

4 3 Seminar and Workshop

Seminar talk program of Dr A Alvarez Dr R Alvarez and Dr D D Shakya at conference room USAID/Nepal on May 23, 1997

Workshop by Dr A Alvarez and R Alvarez at TU on May, 22, 1997

Slide presentation by Dr Alvarez and discussion on black problem at Blue Star Hotel on May 24, 1997

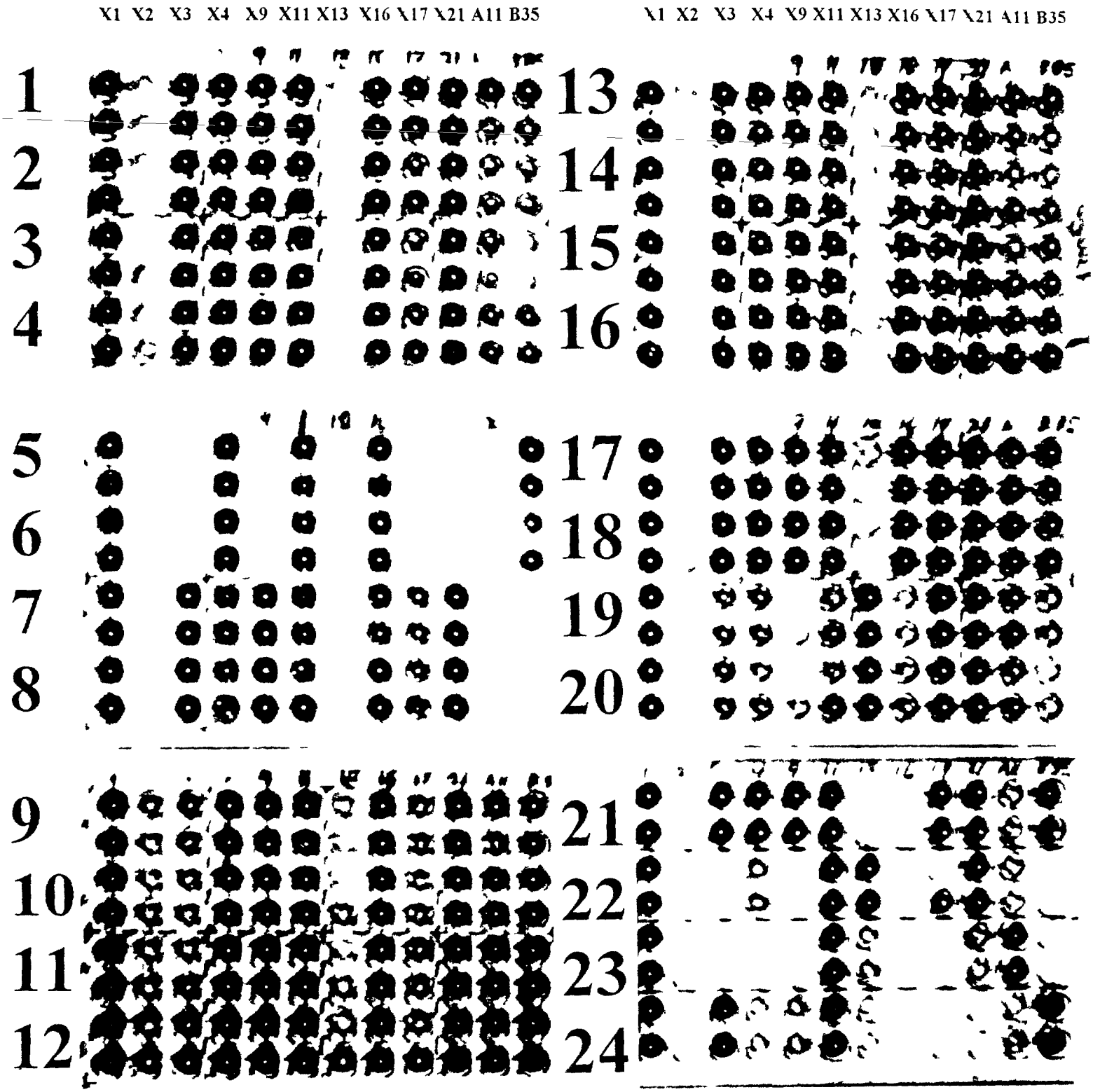
5. Impact

This collaborative project between University of Hawaii and Tribhuvan University has provided a great opportunity to investigate in detail on the situation of Black Rot of crucifers in Nepal The results showing occurrence of Xcc through out the country and sero-typing of Nepalese strains are the first reports of its kind The recommendations made on the basis of the investigation play a great role to control control black rot and

improve crucifer production in the country The study also shows that Nepal has a potentiality for seed cerification program to export Xcc free seeds

The project has a great impact to train a group of the plant pathologists of TU Now we have the team who can work confidently One of the student has made his M Sc dissertation on incidence of black rot with distinction grade under this project Several farmers were made aware of the problem and about its control during the field surveys

Reactions of *Xanthomanas campestris* pv. *campestris* (xcc) strains with monoclonal antibodies



BEST AVAILABLE COPY

US Agency for International Development
Office of Agriculture and Rural Development
USAID/Nepal
Washington, D.C., 20521-6190

CABLE: USAID NEPAL
TELEX 2381-AEKTM-NP

FAX 977-1-272357
TEL 977 1-270-144, 271-425

fax transmission

To: Prof. Dilli Devi Shakya, Executive Director
RECAST, TU

Fax #: 331-964

From: T. David Johnston, ARD

Date: May 19, 1997

Re: Presentation on Plant Bacterial Disease Control

Pages: 4, including this cover sheet

MESSAGE

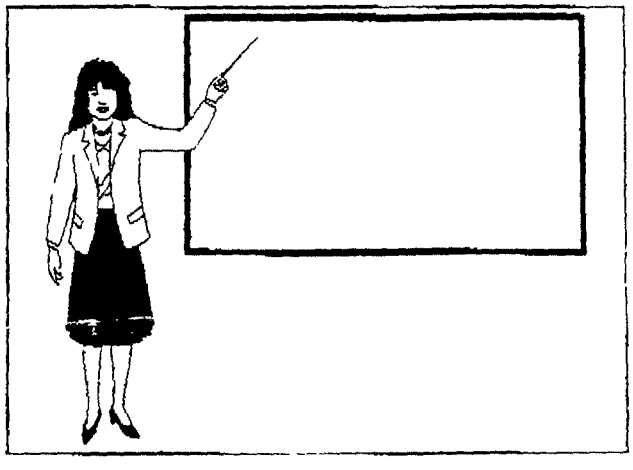
I am sending you a copy of memorandum for your information regarding the presentation on Plant Bacterial Disease Control which is being held at 2 00 PM on Friday, May 23, 1997 in the Conference Room, USAID/Nepal

Thank you

51

MEMORANDUM

TO	See Distribution
FROM	T. David Johnston, Chief, ARD
SUBJECT	Presentation on Plant Bacterial Disease Control
DATE & PLACE :	Friday, May 23rd at 2:00 PM, in the main Conference Room, USAID/Nepal, Rabi Bhawan



Professor Anneal Alvarez, Plant Pathologist of the University of Hawaii and Professor Dill Devi Shakya Executive Director of the Research Center for Applied Science and Technolog (RECAST), Tribhuvan University have been working on control of bacterial diseases in plar with funding from USAID's Program for Scientific and Technological Cooperation (PSTC) They will make a presentation on control of Plant Bacterial Diseases which is based upon their work in Nepal and Hawaii with funding from their PSTC grant

The meeting will last 1 hour including questions and answers Refreshmen will be served following the presentation

We are looking forward to receiving nominatio of individuals from your organization to participate in this presentation by Thursday, May 22 1997 at following numbers

Telephone No 270-144, 272-271 and 271-4[illegible] Ext 223
Fax No 272-357

Thank you

52

BEST AVAILABLE COPY

DISTRIBUTION:

Dr Raghav Dhoj Pant, Member, NPC
Mr Mohand Dev Pant, Secretary, MOA
Mr. Jagadish C Gautam, Executive Director, NARC
Dr Tej Bahadur K C , Dean, IAAS
Dr Tika Adhikary, Plant Pathalogist, IAAS
Ms Carroll Long, Resident Representative, UNDP
Mr Richard T Wurster, Representative, FAO
Mr Marshuk Ali Shah, Resident Chief, ADB
Mr Joe Manickavasagam, Resident Representative World Bank
Mr Leif Christensen, Minister Counsellor, DANIDA
Mr Michael Alcock, First Secretary BTCO
Mr John Abington, Director, Hill Agriculture Research Project
Mr Masao Watanabe, Resident Representative, JICA
Dr Rabi Dev Shakya, Acting Managing Director AEC
Seed Entrepreneur Association, c/o AEC
Charles Drilling, A/Director
Molly Gingerich, SO2
Jane Nandy SO3
Amanda Levenson, FM
Christa Skerry PPD
All ARD Staff

53

BEST AVAILABLE COPY

Bacterial diseases of vegetable crops and their control.

Dr. Anne M. Alvarez, University of Hawaii

Seed - borne bacterial diseases of vegetable crops cause substantial crop losses world-wide and hinder the development of the vegetable seed industry Studies on transmission and survival of pathogenic bacteria were undertaken in a collaborative project between Tribhuvan University and the University of Hawaii to provide the essential background information needed for disease control. Methodologies for detecting specific strains of seed - and soil-borne bacteria were developed using specific monoclonal antibodies and serological assays for identification of pathogens In addition, we produced genetically modified bioluminescent strains to use as "tagged" bacteria in survival studies Uses of these methods in production of pathogen - free seed will be discussed Applications for future research and improvement of disease control measures for rice and vegetable production also will be described

BEST AVAILABLE COPY