Eastern Illinois University

The Keep

Masters Theses

Student Theses & Publications

1-1-2004

Pathogenicity Of Xanthomonas Axonopodis Pv. Glycines, The Causative Agent Of Bacterial Pustule In Soybeans

Lopa D. Goradia

Eastern Illinois University

This research is a product of the graduate program in Biological Sciences at Eastern Illinois University. Find out more about the program.

Recommended Citation

THESIS REPRODUCTION CERTIFICATE

TO: Graduate Degree Candidates (who have written formal theses)

SUBJECT: Permission to Reproduce Theses

The University Library is receiving a number of request from other institutions asking permission to reproduce dissertations for inclusion in their library holdings. Although no copyright laws are involved, we feel that professional courtesy demands that permission be obtained from the author before we allow these to be copied.

PLEASE SIGN ONE OF THE FOLLOWING STATEMENTS:

Booth Library of Eastern Illinois University has my permission to lend my thesis to a reputable college or university for the purpose of copying it for inclusion in that institution's library or research holdings.

_____   _____
Author's Signature   Date

I respectfully request Booth Library of Eastern Illinois University **NOT** allow my thesis to be reproduced because:

_____

_____

_____

_____   _____
Author's Signature   Date

This form must be submitted in duplicate.

Pathogenicity of <u>Xanthomonas</u> <u>axonopodis</u> pv.

<u>glycines</u>, the causative agent of bacterial

pustule^(TITLE) in soybeans

BY

Lopa D. Goradia

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Master of Science

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

2003

YEAR

I HEREBY RECOMMEND THAT THIS THESIS BE ACCEPTED AS FULFILLING
THIS PART OF THE GRADUATE DEGREE CITED ABOVE

1-5-04

DATE

Steven L. Daniel

THESIS DIRECTOR

1/5/04

DATE

DEPARTMENT/SCHOOL HEAD

Pathogenicity of *Xanthomonas axonopodis* pv. *glycines*, the causative agent of bacterial pustule in soybeans

Lopa D. Goradia

Department of Biological Sciences

Eastern Illinois University

Charleston, Illinois 61920

Abstract

Xanthomonas axonopodis pv. *glycines* is the casual agent of bacterial pustule of soybean (*Glycine max* [L.] Merr). It is one of the most prevalent bacterial diseases in many soybean-growing areas, especially in Brazil, China and India where it can cause yield losses of up to 40%. The pathogen is spread by wind-blown rain and survives in crop debris and seed. It infects through stomata and wounds on soybean leaves and causes hypertrophy of host cells. Four isolates of *X. axonopodis* pv. *glycines* were used in this study. Two isolates were isolated from UIUC soybean fields, one was obtained from ATCC, and the last was obtained from the University of Florida. Metabolic fingerprinting and SDS-PAGE were used to compare isolates. Fingerprinting showed that the *X. axonopodis* pv. *glycines* UIUC-1 was different than the other three isolates in utilization of substrates. A greenhouse assay was developed to evaluate the factors that affect the pathogenicity of *X. axonopodis* pv. *glycines* UIUC-1. *Xanthomonas axonopodis* pv. *glycines* UIUC-1 grew well in potato dextrose broth for 8 h at 25°C. *Xanthomonas*

axonopodis pv. *glycines* UIUC-1 was more aggressive on susceptible soybean cultivar than the other three isolates of *X. axonopodis* pv. *glycines*. A wide variety of Roundup ready soybean cultivars were screened for resistance to *X. axonopodis* pv. *glycines* UIUC-1. Greenhouse tests showed that about 25% of the Roundup ready soybean cultivars were susceptible to *X. axonopodis* pv. *glycines* UIUC-1. This indicates commercial soybean companies may not be maintaining resistance and that there is potential for bacterial pustule to cause significant loses in soybean yield to the farmers.

Acknowledgements

Table of Contents

List of Tables

List of Figures

Introduction

Soybean, *Glycine max (L.)* Merr., is a legume that grows in tropical, subtropical and temperate climates and it belongs to the bean family, *Fabaceae* (Sinclair et al., 1999). According to The Food and Agricultural Organization of the United Nations (FAO), over 160 million tons of soybeans were produced in 2000. The leading producer, the USA, accounted for 42% of the total soybeans produced. The next largest producers, Argentina and Brazil, had shares of 24% and 16%, respectively while China yielded 8% of the soybeans produced. In the USA, the production of soybeans is concentrated in the Midwest and Mississippi valley (Soybean Almanac, 2002).

Soybeans are susceptible to several hundred pathogens, some of which are economically important. Fungal pathogens usually predominate, but bacterial, nematode and viral pathogens occur as well (Kucharek, 2001). The most common bacterial diseases of soybeans are bacterial blight and bacterial pustule (Sinclair, 1999).

Bacterial pustule is caused by the bacterium *Xanthomonas axonopodis* pathovar (pv.) *glycines*. Erwin Smith who isolated a yellow bacterium from soybeans may have observed the disease as early as 1902 (Hartwig and Lehman, 1951). The earliest definitive recognition of bacterial pustule came in 1922 when Hedges described the disease and isolated the organism (Hartwig and Lehman, 1951). It is most prevalent in soybean-growing areas, especially Australia, China, India, Japan, Korea, Sudan, and USA (Sinclair and Backman, 1989).

X. axonopodis pv. *glycines* is a gram-negative, aerobic, rod-shaped bacterium in the family Pseudomonadaceae (Bradbury, 1984). It was previously known by several names: *Pseudomonas glycineum* (Coerper), *Pseudomonas glycines* (Nakano),

1

Xanthomonas glycines (Magrou and Prevot), *Xampestris campestris* pv. *glycines* (Dye), and finally *X. axonopodis* pv. *glycines* (Vautertin et. al.). Colonies on beef infusion agar are pale yellow and become deep yellow with age (Sinclair and Backman, 1989; Swings and Civerolo, 1993) (Figure 1). The optimal temperature for growth in potato dextrose broth is 25-30°C (Bhatt and Patel, 1954). It produces extracellular polysaccharides, xanthum gum, auxins and bacteriocins in culture. Extracellular polysaccharides prolong the life of the pathogen under dry conditions and sunlight, increase the survival rate in the laboratory, and support growth in soybean plants (Vorenkevich and Shmanenkova, 1968; Swings and Civerolo, 1993). Weeds such as *Brunnichia cirrhosa* and *Dolichos biflorus* are also natural hosts for *X. axonopodis* pv. *glycines* (Bhatt and Patel, 1954; Jones, 1961; Sinclair, 1989) and *Phaseolus vulgaris, P. lunatus, Lablab purpureus, Arachis hypogaea* and *Pachyrhizus erosus* were infected by artificial inoculation (Sinclair and Backman, 1989; Swings and Civerolo, 1993).

Bacterial pustule occurs principally on the foliage of soybeans. The bacterium overwinters in crop debris, seeds or in the roots (Hartwig and Lehman, 1951). *Xanthomonas axonopodis* pv. *glycines* is spread by splashing water, wind-blown rain, and cultivation when the foliage is wet. The bacterium enters the plant through stomata and wounds and multiplies intercellularly (Sinclair, 1984; Jones and Fett, 1985). The incubation period varies depending on weather conditions, but 7-10 days is the standard incubation period for symptoms to appear (Srivastava and Bais, 1987). A recent study showed that soybean leaf tissues infected by *X. axonopodis* pv. *glycines* have a marked increase in indole-3-acetic acid (IAA) content when compared to noninfected leaf tissues (Kim et al., 2001)
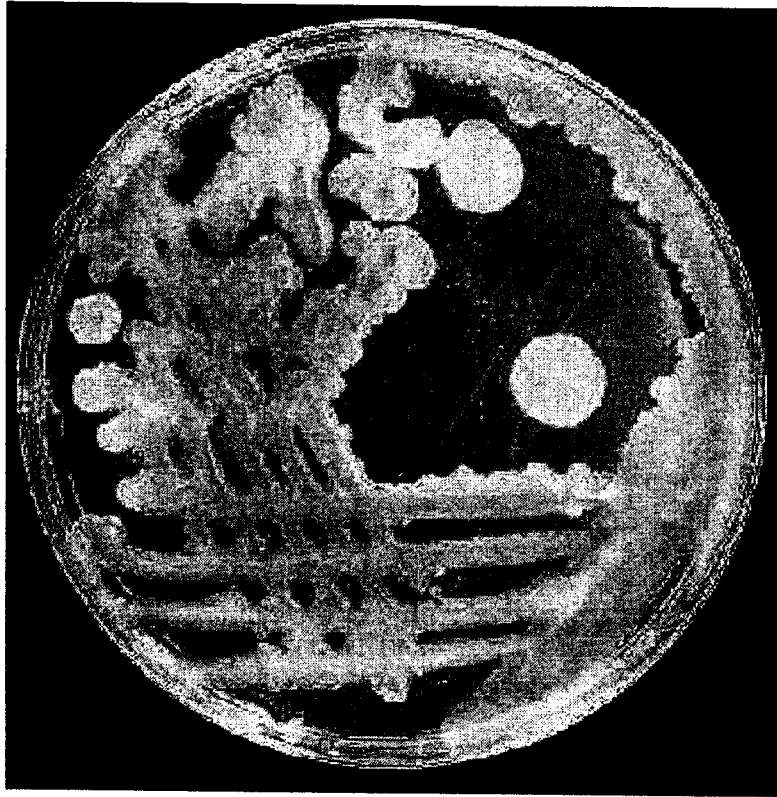
Figure 1. *Xanthomonas axonopodis* pv. *glycines* streaked on potato dextrose

agar   (http://helios.bto.ed.ac.uk/bto/biology/imaghomi.htm)

Young leaves are more susceptible to infection than old leaves. The first symptoms are small, pale green spots (Swings and Civerolo, 1993) with reddish-brown centers on the upper leaf surface (Palmer et. al., 1992) (Figure 2). The central portion of each spot appears slightly raised and develops into a small pustule, especially on the underside of the leaf (Sinclair and Backman, 1989). Several infections on the same leaf produce a large, yellow-to-brown area with small, dark brown spots (Figure 3). Closely spaced infections cause yellowing and death of the intervening tissue and premature shedding of leaves. In later stages, dried, ruptured pustules may be seen on small necrotic areas bordered by narrow yellow halos (Hartwig and Lehman, 1951). The disease symptoms are due primarily to hypertrophy of host mesophyll cells (Groth and Braun, 1986), which leads to premature defoliation of infected plants (Hartwig and Lehman, 1951).

Pustules usually protrude from the underside of the leaf and are composed of enlarged, closely spaced parenchyma cells (Jones and Fett, 1987). It is believed that extracellular proteases, exopolysaccharides, lipopolysaccharides and endoglucanases directly or indirectly play a role in pathogenesis. However, the mechanisms by which the enzymes interact to cause disease in plants are poorly understood (Widjaja et al., 1999). It may become difficult to distinguish bacterial pustule from bacterial blight in the later stages, when the leaves are completely ruptured and dry. Heavy infection of pustule has been found to reduce yield by 4-11% in the USA and up to 20% in Taiwan

Figure 2. Appearance of bacterial pustule on soybean leaves

Figure 3. Appearance of bacterial pustule on soybean leaves at a later stage of development

(http://www.msstate.edu/Entomology/plantpath/field/soybean/bactpustule.htm)

(Srivastava and Bais, 1987). Yields are lowered because of reduced seed size, and under certain environmental conditions, serious economic losses can occur (Oh et al., 1999).

Crop rotation, resistant varieties and burial of crop residues are the most effective methods of disease control. *Xanthomonas axonopodis* pv. *glycines* can also be inhibited by the soybean isoflavonoids glyceollin and coumestrol (Fett and Osman, 1982). A very high level of resistance to bacterial pustule disease is conferred by a recessive gene, designated *rxp* (Bernard and Weiss, 1973) that was originally found in soybean cultivar CNS. This resistance has endured for more than 30 years in spite of the fact that it is the only widely used source of resistance (Bernard and Weiss, 1973; Oh et al., 1999). Soybean cultivars carrying the *rxp* gene are resistant to the bacterium and rarely become infected under natural conditions in the field (Chamberlain, 1962). The *rxp* gene confers resistance, at least in part, by increasing the number of bacterial cells necessary for infection rather than by restricting the pathogen within host tissues (Groth and Braun, 1986). The number of pathogen cells required to initiate pustules is six times greater in resistant cultivars than in susceptible cultivars. Furthermore, genotypes having the *rxp* gene show reduced intensity of symptoms with higher concentrations of inoculum when compared to susceptible genotypes under controlled conditions (Groth and Braun, 1986; Manjaya and Pawar, 1999). Molecular pedigree analysis has also shown that the *rxp* gene is inherited from the ancestral cultivar CNS. The *rxp* gene is linked to the malate dehydrogenase locus and has been mapped on Linkage Group D2 (Narvel et al., 2001).

Many greenhouse inoculation experiments have been successfully used to induce bacterial pustule in soybean seedlings. In 1928 Lehman and Woodside inoculated the unifoliate leaves by rubbing the upper and lower surfaces with heavy suspensions of *X.*

campestris pv. *glycines* (Chotiyarwong, 1985). Chamberlain (1962) forcibly sprayed inoculum against the underside of the leaf with an atomizer attached to a compressed air line until water soaking of leaf tissue was observed. Patel et al. (1972) inoculated plants by spraying the leaves with a bacterial suspension in a Gator-rocking sprayer. All three methods of inoculation resulted in pustule formation on soybean leaves.

Attempts at differentiating pathovars of *X. campestris* by methods other than pathogenicity have included rRNA- DNA and DNA-DNA hybridizations, serology, phage typing, and comparison of profiles resulting from plasmid and chromosomal DNA restriction enzyme digests, protein electrophoresis, and gas chromatography of fatty acids (Lazo et al., 1987). Swings and Civerolo found that protein and biochemical tests demonstrated differences between strains, but were not useful for typing strains of a pathovar. The Biolog method (a microplate system based on the ability of an organism to differentially utilize 95 different carbon sources) has been used to analyze 280 strains belonging to all *Xanthomonas* species and 40 pathovars of *X. campestris* and *X. oryzae*. Although each of the *Xanthomonas* species could be separated, only few *X. campestris* pathovars could be represented as homologous subgroups (Swings and Civerolo, 1993). Lazo et al. (1987) investigated the degree of genetic variation using restriction fragment length polymorphism (RFLP) among 93 strains (consisting of 26 pathovars) of *X. campestris*. They found significant variation among strains within a given pathovar. They concluded that RFLP analysis is suitable for differentiating pathovars and might help to resolve the taxonomy of *Xanthomonas* (Swings and Civerolo, 1993).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has been used to study total protein profiles. However, this technique does not apparently

differentiate pathovars. Vauterin et al. (1991) analyzed SDS-PAGE protein patterns of 307 *Xanthomonas* strains comprised of 27 isolates of *X. campestris,* and showed fairly high similarity values between clusters of *Xanthomonas* species and *X. campestris* pathovars and that most xanthomonads displayed a common SDS-PAGE profile. A number of pathovars, including *X. campestris,* have obvious homogenous entities on the basis of protein electrophoresis (Vauterin et al., 1991). Within *X. campestris,* five pathovars from leguminous hosts, (i.e., *X. campestris* pvs. *phaseoli, cajani, vignicola, alfalfae* and *glycines*) displayed very similar protein profiles (Swings and Civerolo, 1993).

Bacterial pustule in many parts of the world is considered a major soybean disease. In the USA, resistance to bacterial pustule has been developed by traditional breeding using the *rxp* gene. There have been no major epidemics of bacterial pustule in the USA, but it has been observed occasionally in commercial fields. It is not known if the new commercial soybean cultivars with the Roundup ready gene all carry resistance to bacterial pustule.

Objectives

The objectives of this study were to:

1. Evaluate, via greenhouse analysis, factors which impact the pathogenicity of *X. axonopodis* pv. *glycines* on soybeans (e.g., host specificity, strain variability, and nutritional factors);

2. Compare isolates of *X. axonopodis* pv. *glycines* using biochemical (Biolog plates) and molecular techniques (SDS-PAGE and RFLP) analyses;

3. Screen a wide variety of Roundup ready soybean cultivars for resistance to *X. axonopodis* pv. *glycines* via greenhouse and field analyses.

As a result of this study, a better understanding of bacterial pustule, factors that cause the disease, and the aggressiveness of various isolates of *X. axonopodis* pv. *glycines* will be determined. Molecular and biochemical techniques like metabolic fingerprinting and RFLP analyses will help determine if there are any differences among various isolates of *X. axonopodis* pv. *glycines*. The screening of various soybean cultivars will allow us to determine if the cultivars obtained from various seed companies are still resistant to *X. axonopodis* pv. *glycines*. This may ultimately help in preventing the future emergence of bacterial pustule as a serious soybean disease in the USA and elsewhere.

Materials and Methods

Bacterial isolates. Isolates used in this study are listed in Table 1. All isolates were cultured at 25°C on potato dextrose agar (PDA, Difco) plates and stored on PDA slants (7 ml PDA in 16 x 150 mm tubes) at 4°C.

Xanthomonas axonopodis pv. *glycines* UIUC-1 and *X. axonopodis* pv. *glycines* UIUC-2 were isolated from soybean leaves of PI 520.733 and Spencer (susceptible varieties), obtained from UIUC experimental fields. The area of the leaf with pustules was cut with a sterile scalpel and transferred to sterile distilled water (dH₂O). The leaf was macerated to form a homogenized mixture and a loopful was streaked on sterile PDA plates. The plates were incubated for 48 h at 25°C. Isolated colonies were picked and restreaked on PDA until axenic cultures were obtained. Cultures were collected and sprayed on susceptible cultivar (PI 520.733) to determine if the isolates caused pustule. Gram staining was also done to confirm if they were gram-negative rods.

Viability testing. Viable counts of *X. axonopodis* pv. *glycines* UIUC-1 were done at 4 h, 8 h, and 12 h intervals on PDA plates using a ten-fold dilution scheme to determine the number of viable cells at different phases of growth. Plates were incubated for 72 h at 25°C and then colonies were counted. Duplicate plates were used at each dilution and the experiment was replicated twice.

Table 1. Bacterial isolates used in this study

Species	Isolate	Source[a]
Xanthomonas axonopodis pv. *glycines*	UIUC-1	This study
Xanthomonas axonopodis pv. *glycines*	ATCC 17915	ATCC
Xanthomonas axonopodis pv. *glycines*	UIUC-2	This study
Xanthomonas axonopodis pv. *glycines*	UF	UF
Xanthomonas campestris pv. *campestris*	-	UF
Pseudomonas fluorescens	-	EIU

[a] ATCC, American Type Culture Collection; EIU, Eastern Illinois University; UF, University of Florida

Standardization of a greenhouse inoculation assay

A standardized greenhouse inoculation assay was developed to determine the pathogenicity of *X. axonopodis* pv. *glycines* isolates. Standardization was determined by testing growth phases, media, and number of cells sprayed using *X. axonopodis* pv. *glycines* UIUC-1.

Soybeans. Two soybean cultivars were used in this study: a susceptible cultivar, PI 520.733 and a resistant cultivar Williams 82 W 82. Plants were grown in trays with Universal soil mix and vermiculite for 2 weeks at 25 °C. *Xanthomonas axonopodis* pv. *glycines* UIUC-1 was used to confirm the known resistant and susceptible reaction of the cultivars against bacterial pustule. *X. axonopodis* pv. *glycines* UIUC-1 was streaked on PDA plates and incubated at 25°C for 48 h. The culture was washed off the plate with sterile dH_2O and sprayed on the plants by using an atomizer attached to a compressed airline. Bacterial pustules were observed on the susceptible entry after 7 days. Soybean cultivars which showed signs of bacterial pustule when inoculated with *X. axonopodis* pv. *glycines* (UIUC-1 and UIUC-2) were considered to be susceptible cultivars and cultivars with no signs of bacterial pustule following inoculation were considered as resistant cultivars.

Preparation of bacterial cells for plant inoculation. Cells were harvested by centrifugation (11,325 g for 15 mins at 20°C, Beckman J2-HS centrifuge) and washed twice with sterile distilled water (dH_2O). The inoculum was adjusted to an optical density (O.D. at 600 nm) of 0.3 (~10^8 viable cells) with sterile dH_2O for all experiments and 20 ml of the inoculum was sprayed on a total of 16 plants of PI 520.733.

Inoculation technique. Prior to inoculation, trifoliolate leaves were damaged five times by using a perforation needle (to mimic natural wounding). The inoculum was then sprayed on the leaves by using an atomizer attatched to a compressed air line (Figure 4). The results were recorded 7 days later by counting pustules on infected trifoliolate leaves.

X. **axonopodis** pv. **glycines.** The following parameters were evaluated with *X. axonopodis* pv. *glycines* UIUC-1 using the standard greenhouse assay. Each experiment was duplicated.

(i) Live, dead and spent cultures. This experiment was conducted to see if spent culture or dead (autoclaved) cells play any role in pustule development. Inoculum of *X. axonopodis* pv. *glycines* UIUC-1 was prepared by transferring 10 ml of a freshly grown culture to a PDB flask (100 ml/ flask) and grown for 8 h at 25°C on a shaker at 240 rpm. The cells were harvested by centrifugation and the spent culture medium was saved for further analysis. The cells were washed twice with sterile dH_2O and the inoculum was divided into two parts. One part was autoclaved for 25 min at 15 PSI and the second part consisted of viable cells. All three parts (live, dead and spent culture) were sprayed on PI 520.733 using the techniques described above.

(ii) Agar vs. broth culture. This experiment was carried out to see if cells of grown on PDA (solid growth medium) or on PDB (liquid growth medium) caused more pustules on inoculated plants. A loopful of *X. axonopodis* pv. *glycines* UIUC-1 was transferred from PDA plates to PDB flasks to obtain cells from broth. Three transfers were carried out in PDB every 48 h before cells were used for this experiment. To prepare inoculum, 10 ml of a freshly grown culture was transferred to a PDB flask and

Figure 4. Inoculation of susceptible variety PI 520.733 with *Xanthomonas axonopodis* pv. *glycines* in the greenhouse

incubated for 8 h at 25°C on a shaker at 240 rpm. The cells were harvested and inoculum was prepared as above. Cells of *X. axonopodis* pv. *glycines* UIUC-1 from agar were obtained by streaking a loopful of culture on PDA and incubating it for 48 h at 25°C. The inocula were then prepared and sprayed according to the technique described above.

(iii) Impact of culture media. Three different media were tested: PBD, soytone peptone broth (SPB, 2% Soytone peptone [Difco]), and undefined glucose medium (UGM). UGM consisted of the following: 57.4 mM K_2HPO_4; 151.4 mM $(NH_4)_4SO_4$; 73.5 mM KH_2PO_4; 154.1 mM NaCl; 20.3 mM $MgSO_4$; 0.58 mM sodium nitrilotriacetate; 180 μM $FeSO_4$; 40 μM $CuSO_4$; 73 μM $ZnCl_2$; 59 μM $MnSO_4$; 21 μM Na_2MoO_4, 0.1 % yeast extract and 50 mM glucose. All three media were inoculated with 10 ml of starter culture. The flasks were incubated at 25°C for 8 h at 240 rpm. Each culture was transferred three times prior to use in this experiment. The inocula were then prepared and sprayed on PI 520.733 according to the technique described above.

(iv) Impact of growth phase. Three PDB flasks were inoculated with 10 ml of freshly grown starter culture and shaken at 240 rpm at 25°C for 4 h, 8 h, and 12 h respectively. Based on growth analysis, 4 h of incubation represented lag phase, 8 h of incubation represented mid-log phase, and 12 h of incubation represented late-log phase. Cells were harvested at different time intervals by centrifugation and bacterial cells were prepared as mentioned above for inoculation. The inoculum was sprayed on PI 520.733 as described above.

Comparison of *X. axonopodis pv. glycines* isolates

Pathogenicity of different isolates of X. axonopodis pv. glycines. All six bacterial isolates (Table 1) were tested for pathogenicity using the standard greenhouse inoculation assay. All the isolates were grown in PDB for 8 h at 25°C and shaken at 240 rpm. The culture was centrifuged and cells were washed twice with sterile dH$_2$O. The inoculum size was adjusted to an O.D of 0.3 and sprayed on the susceptible variety by using an atomizer attached to a compressed air line. The results were read after 7 days by counting the number of pustules on heavily infected trifoliolate leaves.

Growth in different media. All the isolates (Table 1) were streaked on PDA and incubated at 25 °C for 48 h. A loopful was then transferred to PDB flasks (100 ml/flask), SPB flask and UGM flask. The *X. axonopodis* pv. *glycines* isolates were transferred three times in PDB, SPB and UGM before growth measurements were made. To evaluate growth, 10 ml of the freshly grown culture was transferred to sterile media and flasks of PDB, SPB, and UGM and incubated for 12 h at 25°C at 240 rpm. Optical density readings were taken every hour up to 12 h using a spectrophotometer set at 600 nm. Three flasks were used for each growth medium and the readings were averaged. Sterile media served as the blank. Data was plotted using Sigma Plot 8.0 (SPSS Inc.).

Biochemical fingerprinting. All bacterial strains (Table 1) were grown on PDA at 25°C for 48 h. Cells were removed using a sterile swab so as to prevent any carry over of nutrients from agar medium into the GN-GP inoculating fluid. A homogenous, clump-free suspension was prepared and the density adjusted to 52% using a spectrophotometer. The suspension was poured into a sterile multichannel pipette reservoir. An eight-channel repeating pipettor was used to inoculate the wells (150 µl of the suspension was

inoculated in each well) of GN-GP Biolog plates (Biolog INC.). The plates were incubated at 25°C for 72 h. All the Biolog plates were read at 595 nm (measures substrate oxidation) and 750 nm (measures growth of organism) on Day 1 (0 h) and Day 3 (72 h) using the KC4 software (BIO-TEK). The 72 h reading was subtracted from 0 h reading. The data was then statistically analyzed by cluster analysis using SPSS software. Any reading less than or equal to 0.01 was designated as 0 and any reading above 0.01 was designated as 1.

SDS-Page of whole-cell proteins. Cultures were grown in flasks containing 100 ml PDB for 48 h at 25°C. Cells were harvested by centrifugation at 11,323 g for 15 min and washed twice with sterile dH_2O. The pellet was resuspended in sterile dH_2O. The pellet was transferred to an Eppendorf tube and sonicated six times with a sonicator (BIOSONIK III) at 30% power for 30 sec with 1-min intervals between each sonication. The tubes were then spun in a microcentrifuge at 14,000 rpm for 5 min to remove cell debris. Following centrifugation, the supernatant was removed and analyzed for protein content. To determine the amount of soluble protein present in the cell extract, a 250 μl aliquot of the supernatant was added to 750 μl water and 2.5 ml (1:5 diluted) Bradford reagent (Sigma). The protein concentration was measured using Shimadzu UV Probe (Shimadzu UV/UIS/NIR 3100 spectrophotometer, Version 1.10 software) at 595 nm.

For SDS-PAGE analysis, samples were prepared so that 50 μg of cell protein was added to each well along with an equal amount of loading buffer (4 ml dH_2O, 1 ml 0.5 M Tris.HCl, 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml 2-mercaptoethanol, and 0.2 ml 0.05% bromophenol blue in ethanol). BioRad low-range SDS-PAGE standards were used as molecular weight markers prepared by adding 10 μl of molecular marker solution to 90

μl water. All samples were heated in Eppendorf tubes in a boiling water bath for 5 min. Ten microliters of the marker sample and the entire cell protein sample was loaded on a polyacrylamide gel with 4-20 % gradient (Cambrex). The buffer chamber was filled with 1X running buffer (10X running buffer made of; 30.3 g tris Base, 144 g glycine, 10 g SDS). The gel was allowed to run at 120 V until the bromophenol blue dye marker reached the bottom of the gel. The gel was stained overnight with coomassie blue (10% w/v coomassie blue, 40% methanol and 10% glacial acetic acid) and then destained (25% ethanol, 8% glacial acetic acid) 3 times at 1 hr intervals on the next day.

Restriction fragment length polymorphism. DNA was extracted from bacterial cultures at logarithmic growth phase. The cultures were grown in PDB for 36 h at 25°C. Cells were harvested by centrifugation at 11,323 g for 15 min and the pellet was washed with sterile dH₂O. The pellet was then resuspended in 5 ml of 50 mM Tris–50 mM EDTA –150 mM NaCl (Lazo et. al., 1987). One ml of 1% sodium dodecyl sulfate (SDS) was added and the sample was heated at 55°C for 1 h. The sample was extracted with 6 ml of phenol-chloroform-isoamyl alcohol (25:24:1) by centrifugation at 12,000 rpm for 5 min in a microcentrifuge. The aqueous layer containing the DNA was transferred to a clean tube, trying not to include the material at the interface (Silhavy et. al., 1984). Phenol-chloroform-isoamyl alcohol was added and the aqueous layer was separated. A 0.8 volume of cold isopropyl alcohol was added and centrifuged at 12,000 rpm for 5 min to obtain a DNA pellet. The supernatant was removed and the pellet was washed 3 times with 70% ethanol by centrifugation at 12,000 rpm for 5 min. The extracted DNA was then allowed to dry in the hood for 2 h to evaporate any alcohol. It was then suspended in 100 μl TE buffer (10 mM Tris, 1 mM disodium EDTA) along with RNase (2 μl) and

allowed to dissolve overnight at 4°C. The quality of DNA was determined using Shimadzu software by calculating the DNA: RNA ratio (260:280 wavelength ratio). The DNA samples were frozen until further use.

The concentration of DNA was calculated before the DNA was used for digestion with restriction endonucleases (*Eco*RI and *Bam*HI, Fisher Scientific). The restriction enzyme (RE) digest was carried out in a sterile Eppendorf tube. The following protocol was used to make the RE digest: Sterile dH_2O, 7.8 μl; RE 10X buffer, 2 μl; Acylated BSA (10 μg/μl), 0.2 μl; DNA (5 μg/μl), 10 μl; Mix by pipetting and then add RE (10 μ/μl), 1.0 μl; Final volume, 20 μl.

The reaction mixture was mixed gently by pipetting and flash spun for a few seconds in a microcentrifuge. The tubes were then incubated at 37°C for 2 h. The reaction was stopped by transferring the tubes to an 85°C water bath and then setting the tubes on ice. Five μl of loading dye was added and 15 μl of the digested DNA samples were loaded onto a 0.7% agarose gel. The gel was run in 1X Tris-borate-EDTA (TBE) buffer at 30V overnight or until the loading dye reached the bottom of the gel. It was then transferred to a glass dish with 250 ml distilled water and 20 μl ethidium bromide (EtBr). The glass dish was then placed on a shaker and the gel was stained for 30 min. Finally the gel was destained in 250 ml distilled water for 15 min. A photograph of the gel was taken.

Soybean variety testing

Round-up ready soybean cultivar testing. Round-up ready soybean cultivars (525 cultivars from the 2001 Variety Information Testing Program) were tested for

resistance to *X. axonopodis* pv. *glycines* UIUC-1. Initial screening of 525 cultivars was completed and a short list of 150 cultivars that appeared susceptible from initial evaluation was screened using the standard inoculation assay. Two plants were used per entry. The plants were allowed to grow for 2 weeks until they reached the first trifoliolate stage. *Xanthomonas axonopodis* pv. *glycines* UIUC-1 was grown in PDB for 8 h at 25°C on a shaker at 240 rpm, centrifuged at 11,323 g for 15 min and then washed with sterile dH_2O. The optical density of the inoculum was adjusted to 0.3 using a spectrophotometer. A perforation needle was used to damage the trifoliolate leaves prior to inoculation. Plants were inoculated by spraying the inoculum using an atomizer attached to a compressed air line. Results were recorded after 7 days by counting the number of pustules on heavily infected trifoliolate leaves. The experiment was duplicated.

Field Study. Two soybean cultivars (Pioneer 93B01 and Golden Harvest 3135) were planted in the field in three randomized complete blocks separated by border rows. They were divided into three groups by levels of inoculation: high, low, and uninoculated. Three weeks after planting, all the plants were damaged and sprayed with 1 liter of inoculum diluted in 7.6 liters of water (Figure 5). Thereafter, the high inoculation group was spray inoculated every week and the low inoculation group was inoculated every other week. As the plants grew tall the inoculum size was increased to 2 liters in 7.6 liters of water. Plants were allowed to grow till they reached maturity. The seeds were collected, 300 seeds were weighed, height, lodging and yield was measured and statistical analysis was performed.

Statistical Analysis. Univariate analysis of variance (ANOVA) using SPSS software (Version 11.5; SPSS Inc.), used to determine significance within experiments. *Post hoc* tests were done to test for differences among test groups. Hierarchical cluster analysis was used to observe relationship among isolates using the Biolog results.

Figure 5. Field inoculation of susceptible and resistant varieties of soybean with *X. axonopodis* pv. *glycines* UIUC-1

Results

Isolation and characterization of *X. axonopodis* pv. *glycines*

Preliminary studies confirmed that *X. axonopodis* pv. *glycines* UIUC-1 and *X. axonopodis* pv. *glycines* UIUC-2 isolated from UIUC fields produced pustule on PI520.733. *Xanthomonas axonopodis* pv. *glycines* UIUC-1 and *X. axonopodis* pv. *glycines* UIUC-2 did not produce any signs of pustule on Williams 82. Gram staining showed that *X. axonopodis* pv. *glycines* UIUC-1 and *X. axonopodis* pv. *glycines* UIUC-2 were gram-negative rods and produced yellow, mucoid colonies on PDA plates. The isolates also grew well in PDB and viable counts of *X. axonopodis* pv. *glycines* UIUC-1 approximated ~10^8 CFU/ml.

Optimization of Greenhouse assay

X. axonopodis pv. *glycines* UIUC-1 cells were analyzed to see if dead cells or spent culture medium played any role in development of pustule. Viable cells of *X. axonopodis* pv. *glycines* UIUC-1 produced pustules on susceptible variety of soybeans whereas the dead cells of *X. axonopodis* pv. *glycines* UIUC-1 and the spent culture medium did not produce pustules. Cells of *X. axonopodis* pv. *glycines* UIUC-1 grown on solid medium (PDA) produced more pustules on PI 520.733 than the cells grown on liquid medium (PDB) (Table 2). However, PDB was used for greenhouse standardization in order to determine the affect of growth phase, culture media and number of cells used for inoculation for pustule development.

A significant difference in the number of pustules produced on PI 520.733 (Table 3) was noted among the three different media used to cultivate *X. axonopodis* pv. *glycines*

UIUC-1. *Post hoc* tests showed that the number of pustules produced by cells grown in SPB were significantly less than the number of pustules produced by cells grown in both PDB and UGM.

In the three phases of growth of *X. axonopodis* pv. *glycines* UIUC-1 examined, cells from the log and late-log growth phases developed more pustules on PI 520.733 than did cells from the lag phase (Table 4). Statistical analysis showed that there was a significant difference among the phases of growth.

Based on these results, the standard greenhouse assay used for further studies consisted of the following:

(1) Potato dextrose broth (PDB) was used as the standard culture medium;

(2) Cultures were incubated for 8 h at 25°C on a shaker at 240 rpm;

(3) Cells were harvested by centrifugation, washed with sterile dH$_2$O and adjusted to an O.D (600 nm) of 0.3 (10^8 CFU/ml);

(4) 20 ml of inoculum was sprayed on 16 plants; and

(5) A susceptible variety of soybean (PI 520.733) was used at its first trifoliolate stage of growth.

Table 2. Pustule development on a susceptible variety of soybean (PI 520.733)

inoculated with cells of *Xanthomonas axonopodis* pv. *glycines* UIUC-1 grown on

solid and liquid media

Type of medium	Pustules per plant (X ± SE)[a]
Solid	46 ± 5
Liquid	28 ± 6

[a] Values represent the mean ± the standard error of 16 plants. $p = 0.4$

Table 3. Average number of pustules on a susceptible variety of soybean (PI 520.733) inoculated with cells of *Xanthomonas axonopodis* pv. *glycines* UIUC-1 grown on potato dextrose agar (PDB), soytone peptone broth (SPB) and undefined glucose medium (UGM)

Growth medium	Pustules per plant (X ± SE)[a]
PDB	$59 \pm 7^{*}$
SPB	$35 \pm 7^{\#}$
UGM	$70 \pm 13^{*}$

[a] Values represent the mean ± the standard error of 16 plants.

*/# Values with different symbols indicate means are significantly different (P < 0.001)

Table 4. Average number of pustules on a susceptible variety of soybean (PI 520.733) inoculated with cells of *Xanthomonas axonopodis* pv. *glycines* UIUC-1 harvested at different phases of growth

Phase of growth	Pustules per plant (X ± SE)[a]
Lag (4 h)	$27 \pm 4^{*}$
Mid log (8 h)	$49 \pm 7^{\#}$
Late log (12 h)	$41 \pm 6^{\#}$

[a] Values represent the mean ± the standard error of 16 plants.
*/# Values with different symbols indicate means are significantly different (P < 0.15)

Comparison of *X. axonopodis* pv. *glycines* isolates

Isolates of *X. axonopodis* pv. *glycines* were tested for pathogenicity on PI 520.733 (Table 5). Based on the number of pustules developed per plant, *X. axonopodis* pv. *glycines* UIUC-1 was more aggressive than the other three isolates. None of the isolates caused pustule development on the resistant variety (except *X. axonopodis* pv. *glycines* UIUC-1 on a few plants). *X. campestris* pv. *campestris* and *P. fluorescens* were also unable to produce pustules on both entries.

Growth of the four isolates of *X. axonopodis* pv. *glycines* was measured in three different media. All isolates of *X. axonopodis* pv. *glycines* showed a similar growth pattern in PDB and SPB. (Figures 6, 7 and 8). The isolates had a prolonged lag phase when grown in UGM.

Metabolic fingerprinting. A dendogram was constructed using cluster analysis to observe similarity among isolates based on substrate oxidation (Figure 9). It showed that *X. axonopodis* pv. *glycines* UIUC-2, ATCC 17915, UF, and *X. campestris* pv. *campestris* formed were closer in similarity to each other whereas, *X. axonopodis* pv. *glycines* UIUC-1 and *P. fluorescens* were different than the other isolates. *Xanthomonas axonopodis* pv. *glycines* UIUC-1 was able to oxidize 42 of the 95 substrates while *X. axonopodis* pv. *glycines* UIUC-2, ATCC 17915, and UF were able to oxidize 80, 74, and 77 of the 95 substrates, respectively. Among the subtrates oxidized were dextrin, glycogen, fructose, fucose, galactose, sucrose, citris acid, lactic acid, maltose, proline, and glycerol.

At 750 nm, it was observed that *X. axonopodis* pv. *glycines* UIUC-1, ATCC 17915, *X. axonopodis* pv. *glycines* UIUC-2 and UF were able to grow on 59, 74, 91, and

77 of the 95 substrates, respectively. All four *X. axonopodis* pv. *glycines* isolates were able to grow on 54 of the 95 substrates. A few of the substrates that supported growth of *X. axonopodis* pv. *glycines* were dextrin, glycogen, tween 40, sucrose, glucose, lactic acid, alanine, propionic acid, citric acid, and malonic acid.

SDS-PAGE. Gel electrophoresis was performed to examine the soluble proteins extracted from bacterial isolates (Figure 10). The gel showed minor differences in protein-banding pattern among the bacterial isolates. No obvious variations were observed among the isolates of *X. axonopodis* pv. *glycines*.

RFLP. Genomic DNA of the bacterial isolates was digested with restriction enzymes *Eco*RI and *Bam*HI and run on agarose gel to separate it according to size. It was not possible to visualize DNA variability among the isolates as it formed smears in the gel instead of separating into bands of various sizes.

Soybean variety testing

Evaluation of Round-up ready soybean cultivars. The susceptible cultivar, PI 520.733 and the resistant cultivar, W82 both showed signs of bacterial pustule. These controls were used as cut-off values to divide the cultivars into three groups namely; highly susceptible (66 cultivars), moderately susceptible (41 cultivars) and less susceptible (44 cultivars (Tables 6, 7 and 8).

Field Study. A significant difference was observed among height, seed weight, and yield of soybean varieties: Pioneer 93B01 and Golden Harvest 3135 but not for lodging of seed quality. Overall, inoculation treatment had a significant effect on height

and yield when compared to the controls (Table 9). But the high and low inoculation treatments did not differ significantly from either of these traits.

Table 5. Average number of pustules on a susceptible variety of soybean (PI 520.733) inoculated with cells of different isolates of *Xanthomonas axonopodis* pv. *glycines*

Isolates	Pustules per plant (X ± SE)[a]
X. axonopodis pv. *glycines* (UIUC-1)	73 ± 9*
X. axonopodis pv. *glycines* (ATCC)	37 ± 5[#]
X. axonopodis pv. *glycines* (UIUC-2)	29 ± 3[#]
X. axonopodis pv. *glycines* (UF)	34 ± 5[#]

[a] Values represent the mean ± the standard error of 16 plants.

*/# Values with different symbols indicate means are significantly different (P < 0.001)
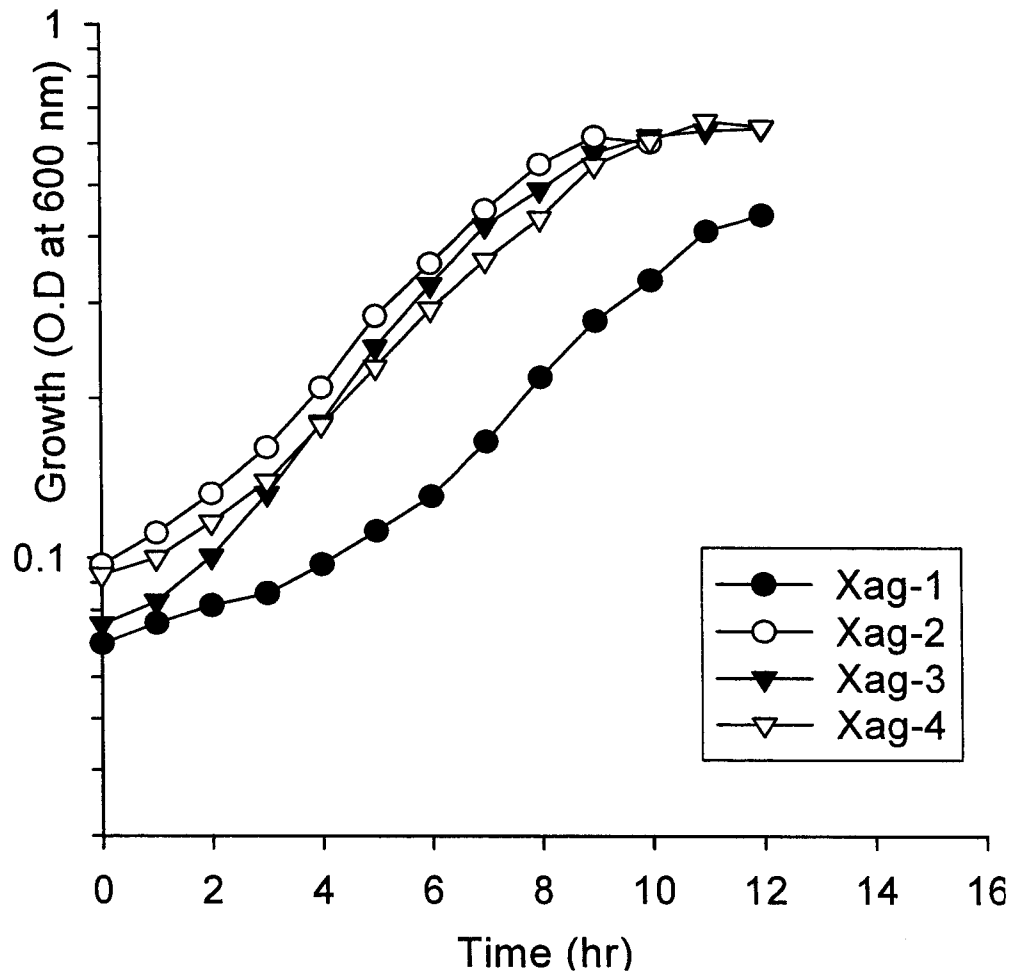
Figure 6. Growth of *Xanthomonas axonopodis* pv. *glycines* (UIUC-1, ATCC 17915, UIUC-2, and UF), *Xanthomonas campestris* pv. *campestris* (Xcc) and *Pseudomonas flourescens* (P.f) in potato dextrose broth (PDB)
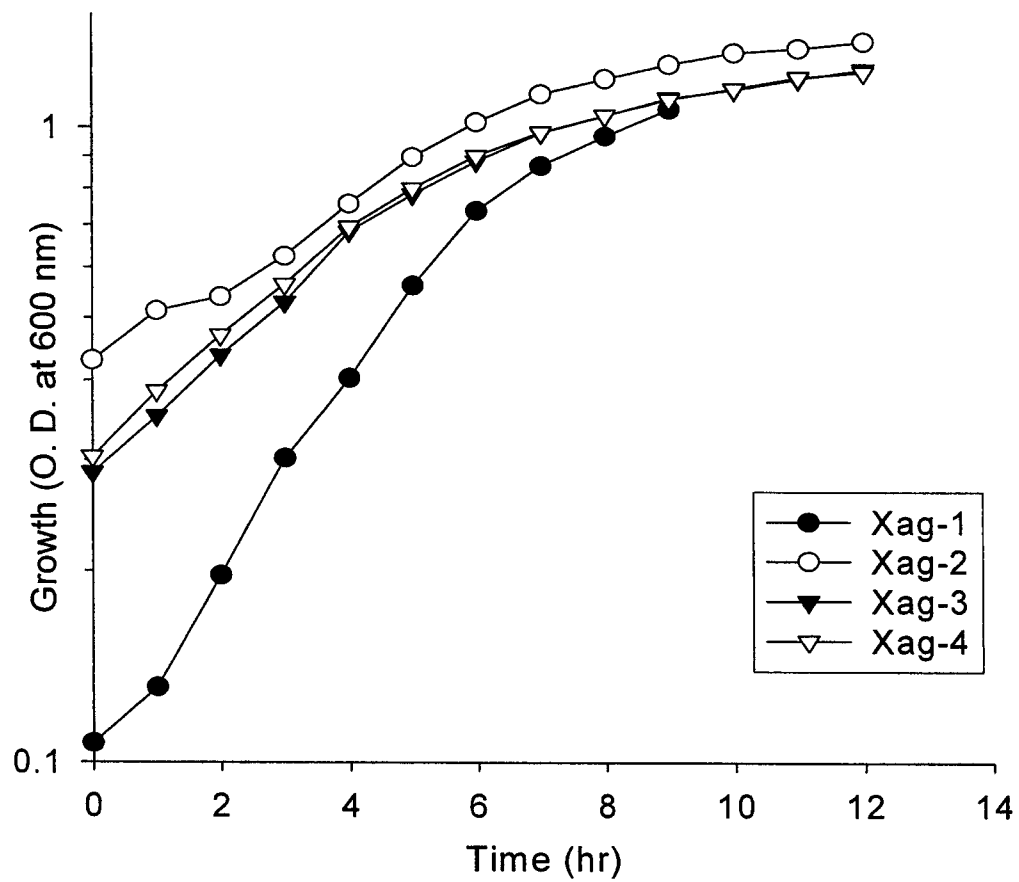
Figure 7. Growth of *Xanthomonas axonopodis* pv. *glycines* (UIUC-1, ATCC 17915, UIUC-2, and UF) in soytone peptone broth (SPB)
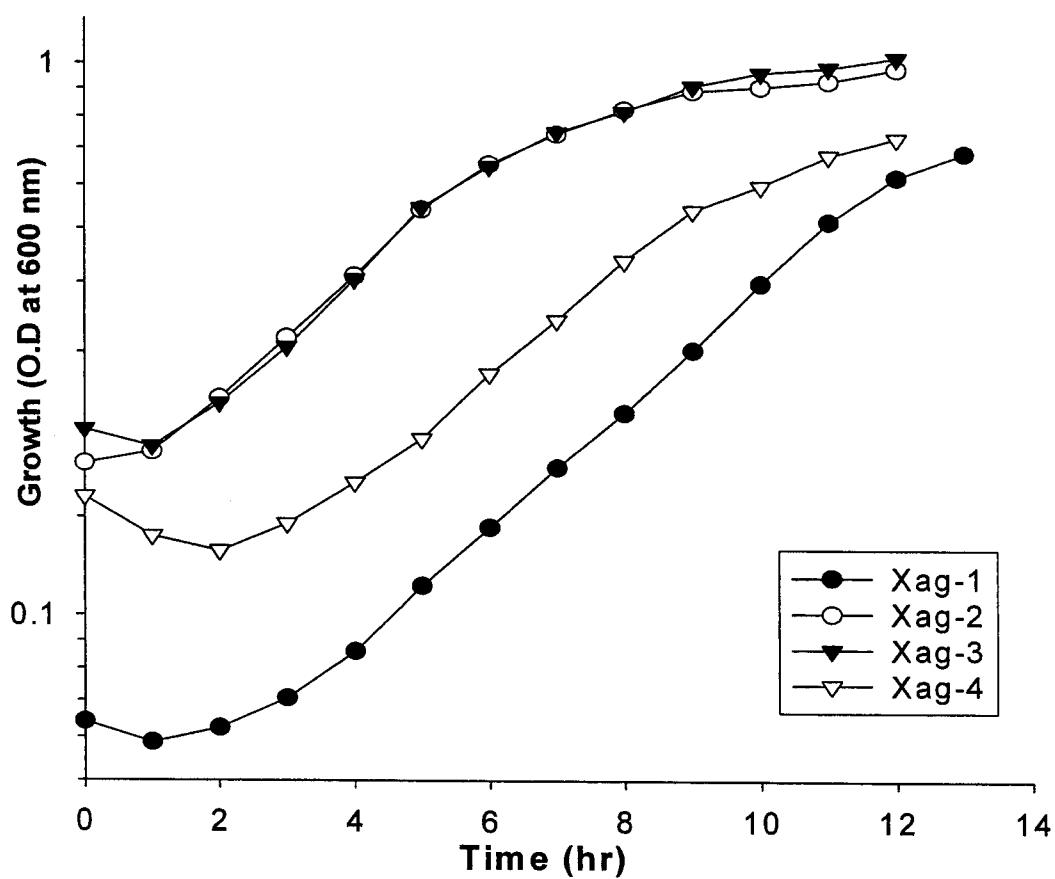
Figure 8. Growth of *Xanthomonas axonopodis* pv. *glycines* (UIUC-1, ATCC 17915, UIUC-2, and UF) in undefined glucose medium (UGM)

```
                            Rescaled Distance Cluster Combine

  C A S E         0         5        10        15        20        25
   Label    Num   +---------+---------+---------+---------+---------+

  UIUC-2     2    ┌──┐
  UF         4    ┤  │
  ATCC       3    ┌──┤
  Xcc        5    ┘
  UIUC-1     1
  P.f.       6
```
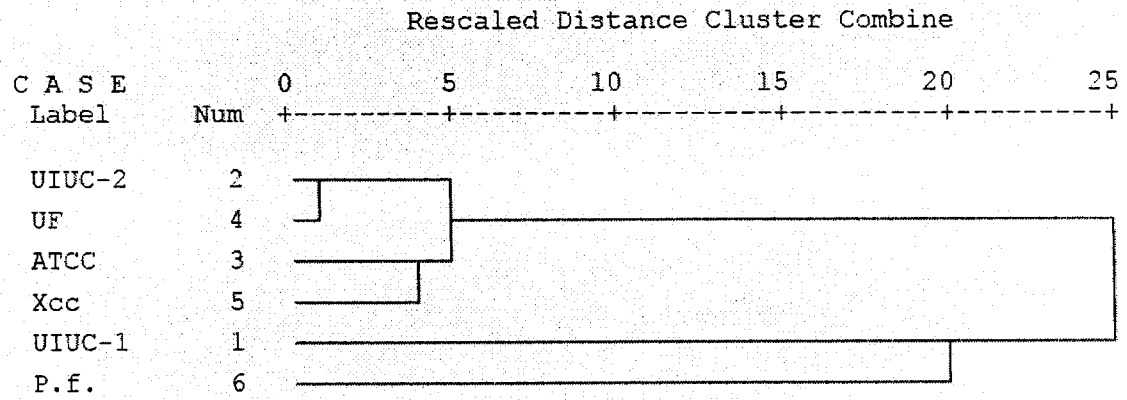
Figure 9.   Dendrogram of *Xanthomonas axonopodis* pv. *glycines* isolates based
on the number of substrates utilized with Biolog plates

36

Lanes 1 2 3 4 5 6 7

MW
(Daltons)

107,000

52,000

36,800

27,200
19,000
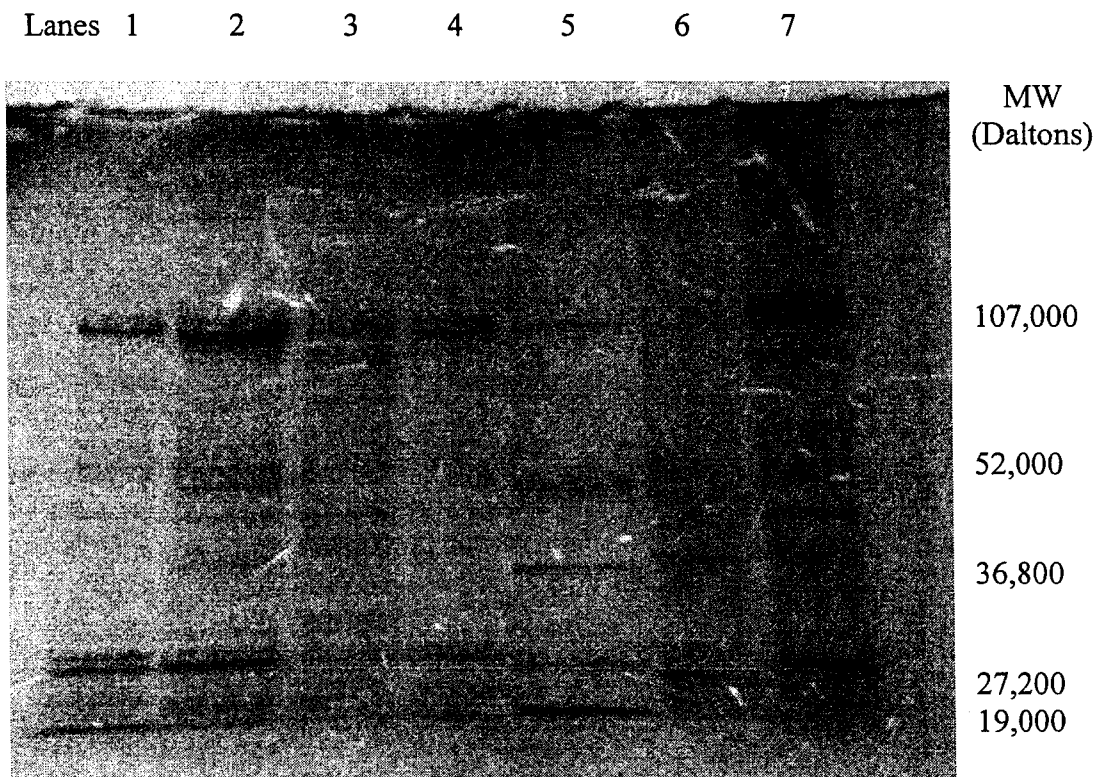
Figure 10. SDS-PAGE analysis of proteins from *Xanthomonas axonopodis* pv. *glycines*

isolates. Lanes 1-4: isolates of *Xanthomonas axonopodis* pv. *glycines* (ATCC 17915, UF,

UIUC-2 and UIUC-1); Lane 5: low molecular weight (MW) marker and Lane 6-7:

Xanthomonas campestris pv. *campestris* and *Pseudomonas fluoroscens,* respectively

Table 6. Reactions of 66 Roundup ready soybean cultivars that were rated as highly susceptible to *Xanthomonas axonopodis* pv. *glycines* UIUC-1

Name	Company	Average # of pustules/plant
DAIRYLAND	DST 2129 RR	161
WILKEN	3402 RR	104
ROESCHLEY	R 4268 RR	94
DELTA & PINE LAND	DPX 4300 RR	90
EXCEL	8254 RR	88
WILLCROSS	EX 292 N	87
SCHILLINGER	211.RW	86
SOUTHERN STATES	RT 4098	86
WILKEN	2582 RR	84
STRIKE	2801 RR	84
FS HISOY	HS 3005	77
DEKALB	DKB 44-51	77
SCHILLINGER	321.RC	76
SUN PRAIRIE	SP 3400 RR	76
DERAEDT	2820 RR	75
TRISLER	TRISOY 3217 RRN	72
BAKER	4505 CRR	71
UNITED SUPPLIERS	US S2709 RR	70
KRUGER	K-444 RR/SCN	69
SOUTHERN STATES	RT 4495 N	68
LATHAM	667 RR	68
BECK	283 RR	67
GOLDEN HARVEST	H 3983 RR	67
STRIKE	3101 RR	65
KRUGER	K-300 RR	65
FS HISOY	HS 3706	64
STINE	2463-4	64
EXCEL	8195 RR	64
MERSCHMAN	CHEROKEE XRR	64
EXCEL	8234 RR	63
STINE	3808-4	63
DAIRYLAND	DSR-322 RR	62
BAKER	4425 CRR	62
DAIRYLAND	DSR-395 RR	62
KRUGER	K-323-3 RR	61
VIGORO	V 272 NRR	61
KRUGER	K-262-2 RR	59
BECK	437 NRR	58
UAP	DG 3370 RR	58
VIGORO	V 363 NRR	58
ASGROW	AG 4403	57
CROW'S	C 48009 RN	57
SIEBEN	2803 RR	57
EXCEL	8395 RR	56
KALTENBERG	KB 244 RR	56
HUGHES	551 RR	56
STINE	3800-4	55
KRUGER	K-330 RR	54
KRUGER	K-330 RR/SCN	52
PIONEER	93B67	51

SCHILLINGER	271.RPW	51
VIGORO	V 442 NRR	51
FS HISOY	HS 4107	51
TRISLER	TRISOY 3017 RRN	50
GOLDEN HARVEST	H 3505 RR	48
HENKEL	SS 2618	48
MWS	285 CRR	48
EXCEL	8410 NRR	47
KRUGER	K-323 RR	46
KRUGER	K-286 RR	46
VIGORO	V 282 RR	45
LATHAM	EX-1097 RR	45
EXCEL	8314 RR	44
MERSCHMAN	RICHMOND VIRR	44
KRUGER	K-282-2 RR	43
STEYER	4410 SCN	41
DAIRYLAND	DSR-228 RR	41
PI 520.733	NA	43

Table 7. Reactions of 41 Roundup ready soybean cultivars that were rated as moderately

susceptible to *Xanthomonas axonopodis* pv. *glycines* UIUC-1

Name	Company	Average # of pustules/plant
PI 520.733	NA	43
PIONEER	94B23	40
UAP	DG 3484 NRR	340
PIONEER	93B85	39
BERGMANN-TAYLOR	BT 400 CR	39
SUN PRAIRIE	SP 3702 NRR	39
HORIZON	H 328 NRR	39
PRAIRIE BRAND	PB-2841 RR	38
KRUGER	K-443-3 RR/SCN	38
UAP	DG 3278 RR	37
LATHAM	EX-787 RR	37
WILKEN	2583 RR	36
KRUGER	K-289 RR	36
DAIRYLAND	DSR-232 RR	35
WILKEN	2691 NRR	35
GOLDEN HARVEST	H 2871 RR	34
KRUGER	K-388+ RR	33
GREAT LAKES	GL 3409 RR	33
FS HISOY	HS 2906	32
UAP	DG 3468 NRR	32
SCHILLINGER	250.RPB	31
WILLCROSS	RR 2242	31
WILKEN	3461 NRR	31
DEKALB	DKB 26-51	30
WILKEN	2568 RR	30
SCHILLINGER	331.RCP	30
SCHILLINGER	281.RIP	29
KRUGER	K-262 RR/SCN	29
WILKEN	WE 944 RR	28
PRAIRIE BRAND	PB-2861 RR	28
WILLCROSS	RR 2331 N	28
BERGMANN-TAYLOR	BT 351 R	26
BIO GENE	BG 4401 NRR	25
KRUGER	K-222+ RR	25
DELTA KING	XTJ 174 RR	24
DEKALB	DKB 23-51	24
DELTA KING	4965 RR	24
DEKALB	DKB 28-51	23
STINE	4202-4	22
FS HISOY	RT 4585	21
MERSCHMAN	CHICKASAW VIIRR	21
STINE	2736-4	21

| KRUGER | K-255 RR | 22 |
| Williams 82 | NA | 19 |

Table 8. Reactions of 44 Roundup ready soybean cultivars that were rated as less

susceptible to *Xanthomonas axonopodis* pv. *glycines* UIUC-1

Name	Company	Average # of pustules/plant
Williams 82	NA	19
WILLCROSS	RR 2321 N	20
WILKEN	2318 RR	18
ASGROW	AG 2703	18
WILLCROSS	RR 2392 N	18
DEKALB	DKB 40-51	18
DELTA KING	4762 RR	17
WILKEN	3498 RR	17
SUN PRAIRIE	XP 3132 RR	16
DAIRYLAND	DSR-272 RR	15
EXCEL	8484 RR	15
KRUGER	K-444-4 RR/SCN	15
SCHILLINGER	310.RP	15
VIGORO	V 393 NRR	15
DAIRYLAND	DSR-290 RR	14
LATHAM	EX-747 RRN	14
FS HISOY	X 2815	14
FS HISOY	RT 3585	13
DEKALB	DKB 45-51	13
MERSCHMAN	ROOSEVELT IIIRR	12
WILKEN	3403 RR	12
UNITED SUPPLIERS	US E3802 RR	12
UNITED SUPPLIERS	US S2101 RR	11
HORIZON	H 379 RR	9
KITCHEN	KSC 3926 CRR	9
WILLCROSS	RR 2350	9
EXCEL	8306 RR	8
KRUGER	K-255-5 RR	8
PRAIRIE BRAND	PB-3404 RR	7
BECK	367 NRR	7
MERSCHMAN	KENNEDY VIRR	6
RENK	RS 310 RR	6
DAIRYLAND	DSR-268 RR	5
FS HISOY	X 2515	5
KRUGER	K-250-1 RR	5
GOLDEN HARVEST	H 3960 RR	5
AGRINETICS	EXCEL 8274 RRN	4
WILKEN	3471 NRR	4
ASGROW	AG 2402	3
WILKEN	3551 NRR	3
MERSCHMAN	SHAWNEE VIIIRR	2
KRUGER	K-252+ RR	2

DELTA KING	5366 RR	2
MIDWEST SEED GEN	GR 3331	2
DEKALB	DKB 32-52	2

Table 9. Effect of *Xanthomonas axonopodis* pv. *glycines* UIUC-1 on soybean growth

and yield in the field

Treatment	Soybean			
	Height (inches)[*]	Weight (grams)	Yield (bushels)*	Lodging
Non-inoculated plots	32	43	50	1.5
Inoculated plots	30	42	43	1.5

Height, weight, yield, and lodging were measured at the time of maturity

* Symbol indicates that the parameters have a significant difference

Discussion

Initial studies involved the isolation of *X. axonopodis* pv. *glycines* (UIUC-1 and UIUC-2) for standardization of greenhouse assay and comparison studies. *X. axonopodis* pv. *glycines* UIUC-1 and *X. axonopodis* pv. *glycines* UIUC-2 are gram-negative rods and formed pale yellow, circular, smooth and slimy colonies on PDA. The isolates were confirmed to be *X. axonopodis* pv. *glycines* by the formation of bacterial pustules on susceptible soybean varieties.

Different media such as Luria-Bertani broth (Heu et al., 2001), yeast extract-dextrose-calcium carbonate agar (Palmer et al., 1992), and potato dextrose agar (Fett et al., 1987) have been used to grow *X. axonopodis* pv. *glycines*. This study showed that all four isolates of *X. axonopodis* pv. *glycines* were also able to grow on three different media – Potato dextrose broth, Soytone peptone broth, and Undefined glucose media.

Results from greenhouse standardization suggest that the plants should be grown for two weeks and inoculated with the standard inoculation method. Experiments show that the most suitable stage of growth for artificial inoculation appears to be during or after the initiation of the reproductive stage (Chotiyarnwong, 1985). Studies have shown that the inoculum size is adjusted to a cell density of 10^8 CFU/ml before spraying the plants (Hwang, 1990). Greenhouse studies have also shown that the most severe infections were obtained when inoculations were made at the time of day when the stomatal openings were the widest (Feaster, 1951). Inoculation techniques used in this study were similar to Jones and Hartwig (1959), by forcibly spraying the cell suspension against the leaf surface with a atomizer attatched to a high pressure.

In this study, pathogenicity testing of *X. axonopodis* pv. *glycines* isolates on a susceptible cultivar of soybeans (PI520.733) showed that some isolates of *X. axonopodis* pv. *glycines* was more aggressive than the others. *Xanthomonas axonopodis* pv. *glycines* UIUC-1 was the only isolate that produced signs of bacterial pustule on a resistant cultivar of soybeans (W82). Of the nine isolates of *X. axonopodis* pv. *glycines*, only isolate 8601 collected from the disease nursery of USDA Soybean Germplasm Collection at Urbana, IL was virulent on Williams 82 (Hwang 1990). This suggests that isolates of *X. axonopodis* pv. *glycines* can cause bacterial pustule on W82.

Metabolic fingerprinting (Biolog) has been used successfully to distinguish pathovars of *X. campestris* (Norman et al., 1997). In this study, *X. axonopodis* pv. *glycines* UIUC-1 was found to differ in its ability to utilize carbon substrates when compared to a known isolate of *X. axonopodis* pv. *glycines* (ATCC 17915). However, a larger number of isolates of *X. axonopodis* pv. *glycines* should be tested using metabolic fingerprinting in order to determine if a difference exists among isolates.

To help discriminate between isolates, SDS-PAGE was used to visualize different protein banding patterns of the isolates. The SDS-PAGE membrane protein profiles of the isolates of *X. axonopodis* pv. *glycines* were essentially identical to each other and thus do not appear to be useful in differentiating isolates of *X. axonopodis* pv. *glycines*. As observed from *Xanthomonas* species and *X. campestris* pathovars, most of the xanthomonads display common SDS-PAGE protein profiles. Highly distinct profiles were only observed in *X. albilineans* and *X. maltophilia* (Vauterin et al., 1991).

Finally, in order to determine if there was variation in DNA sequences among the isolates, RFLP analysis was used. DNA sequence variation was examined by digesting

the DNA with restriction enzymes and directly visualizing the resulting fragments on ethidium bromide-stained gels. Identification of bacterial isolates using RFLP was difficult since large numbers of restriction fragments were produced. Since it was difficult to differentiate between isolates of *X. axonopodis* pv. *glycines* using RFLP, other restriction enzymes or molecular techniques should be utilized. Pulse-field gel electrophoresis (PFGE) which separates large restriction fragments generated by rare-cutting restriction endonucleases might be utilized (Chan and Goodwin, 1999).

It is difficult to discriminate among isolates of *X. axonopodis* pv. *glycines* without extensive pathogenicity testing. Techniques for differentiating *X. campestris* isolates such as monoclonal antibodies, SDS-PAGE, RFLP, and genomic DNA fingerprinting might be used on *X. axonopodis* pv. *glycines* in the future (Norman et al., 1997). However, widespread use of these techniques is limited due to expense, time and the ability to differentiate isolates at the pathovar level.

The variety testing of soybean cultivars in the greenhouse has shown that about 25% of the cultivars were susceptible to *X. axonopodis* pv. *glycines*. Soybean cultivars carrying the *rxp* allele are resistant to *X. axonopodis* pv. *glycines* and are only infected under rare conditions (Palmer et. al., 1992). Further studies are needed to determine if resistance is being maintained in soybean seeds when other isolates of *X. axonopodis* pv. *glycines* are used. Additional field research also needs to be done to look at the possibility of pustule development under natural conditions.

These results suggest that resistance is not being maintained in commercial soybean cultivars, which may lead to an increased incidence of bacterial pustule in the

field, ultimately impacting soybean yields. Further research calls for observing if the *rxp* gene has been inserted in these soybean cultivars.

Literature Cited

Bernard R. L. and Weiss M. G. 1973. Qualitative genetics. In: B. E. Cadwell ed., Soybeans: Improvement, production, and uses. Agron. monogr. 16. ASA, Madison, WI. p 117-146

Bhatt V. V. and Patel M. K. 1954. Comparative study of species of *Xanthomonas* parasitising leguminous plants in India. Indian Phytopathology 7:160-180

Bradbury J. F. 1984. Xanthomonas Dowson 1939. In, Krieg N. R and Holt J. G., ed. Bergey's manual of systematic bacteriology, Vol. 1. Williams & Wilkins, Baltimore Md. p 199-210

Chamberlain D. W., 1962. Reaction of resistant and susceptible soybeans to *Xanthomonas phaseoli* var. *sojensis.* Plant Dis Rep. 46:707-709

Chan J. W. Y. F. and Goodwin P. H. 1999. Differentiation of *Xanthomonas campestris* pv. *phaseoli* from *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* by PFGE and RFLP. European J. of Plant Path. 105: 867-878

Chotiyarnwong A. 1985. Suitable growth stages of soybean for bacterial pustule inoculation. AVRDC Research Intern Report

Feaster C. V. 1951. Bacterial pustule disease in soybeans: artificial inoculation, varietal resistance, and inheritance of resistance. Research Bulletin 487. University of Missouri

Fett W. F. and Osman S. F. 1982. Inhibition of bacteria by the soybean isoflavonoids glyceollin and coumestrol. Physiology and Biochem. 72(7):755-760

Fett W. F., Dunn M. F., Maher G. T., and Maleeff B. E. 1987. Bacteriocins and temperate phage on *X. campestris* pv. *glycines.* Current Microbiol. 16:137-144

Groth, D. E. and Braun E. J. 1986. Growth kinetics and histopathology of *Xanthomonas campestris* pv. *glycines* in leaves of resistant and susceptible soybeans. Phytopathology 76:959-965

Hartwig E. E and Lehman S. G. 1951. Inheritance of resistance to the bacterial pustule disease in soybeans. Agron J. 43:226-229

Heu S., Oh J., Kang Y., Ryu S., Cho S. K., Cho Y., and Cho M. 2001. *gly* gene cloning and expression and purification of glycinecin A, a bacteriocin produced by *X. campestris* pv. *glycines* 8ra. Applied and Environ. Microbiol. 67(9):4105-4110

Horsfall J. G. and Barratt R. W. 1945. An improved system for measuring plant diseases. (Abstr.) Phytopathology 35:655

Hwang I. 1990. *X. campestris* pv. *glycines*: Pathogenicity genes, races, and soybean yield [dissertation]. Urbana (IL): University of Illinois at Urbana-Champaign. 156 p.

Jones J. P. 1961. A weed host of *Xanthomonas phaseoli* var. *sojense*. Phytopathology 51:206

Jones, J. P. and Hartwig, E. E. 1959. A simplified method for field inoculation of soybean with bacteria. Plant Dis. Rep. 43:946

Jones S. B. and Fett W. F. 1985. Fate of *Xanthomonas campestris* infiltrated into soybean leaves: an ultrastructural study. Phytopathology 75:733-741

Jones S. B. and Fett W. F. 1987. Bacterial pustule disease of soybeans: microscopy of pustule development in a susceptible cultivar. Phytopathology 77:266-274

Kim H-S, Park H-J, Heu S. and Jung J. 2001. Possible association of Indole-3-Acetic

Acid production by *Xanthomonas axonopodis* pv. *glycines* with development of pustule disease in soybean. Agric. Chem. Biotechnol. 44(4):173-176

Kucharek T. 2001. Some common soybean leaf and stem diseases. Plant Pathology Fact Sheet. University of Florida p.16

Lazo G. R., Roffey R., and Gabriel D. W. 1987. Pathovars of *Xanthomonas campestris* are distinguishable by restriction fragment-length polymorphism. Int'l J. of Sys Bact. p. 214-221

Manjaya J. G., and Pawar S. E. 1999. New genes for resistance to *Xanthomonas campestris* pv. *glycines* in soybean [Glycine max (L.) Merr.] and their inheritance. Euphytica 106:205-208

Narvel J. M., Jakkula L. R., Phillips D. V., Wang T., Lee S.-H. and Boerma H.R. 2001. Molecular mapping of *rxp* conditioning reaction to bacterial pustule in soybean. The J. of Heredity 92(3): 267-270

Norman D. J., Yeun J. M. F. and Hodge N. C. 1997. New disease on ornamental asparagus caused by *Xanthomonas campestris* in Florida. Plant Dis. 81: 847-850

Oh C., Heu S. and Choi Y.-C. 1999. Sensitive and pathovar-specific detection of *Xanthomonas campestris* pv. *glycines* by DNA hybridization and polymerase chain reaction analysis. Plant Pathol. J. 15(1):57-61

Palmer R. G., Lim S. M. and Hedges B. R. 1992. Testing for linkage between the *rxp* locus and nine isozyme loci in soybean. Crop Sci. 32:681-683

Patel P. N., Jindal J. K. and Rao M. V. B. 1972. Studies on resistance in crops to bacterial diseases in India. III. Pustule resistance in soybean. Indian Phytopath. 25:168-171

Silhavy T. J., Berman M. L. and Enquist L. W. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Sinclair J. B., ed. 1984. Compendium of Soybean Diseases. Minnesota: APS Press p 4-5

Sinclair J. B. and Backman P. A., ed. 1989. Compendium of Soybean Diseases. 3rd ed. Minnesota: APS Press

Sinclair J.B. 1999. Bacterial pustule. In, Hartman G. L., Sinclair J. B., and Rupe J. C, editors, Compendium of soybean diseases. 4th ed. Minnesota: APS press p 6-7

Soybean Almanac. 2002 (http://www.unitedsoybean.org/soystats2002/worldstats/index.html)

Srivastava S. S. L. and Bais B. S. 1987. Survival and virulence of *Xanthomonas campestris* pv. *glycines* causing leaf pustules of soybean. Indian J. of Plant Pathol. 6(1): 22-23

Swings J. G. and Civerolo E. L. 1993. Xanthomonas. Chapman & Hall. London

Vauterin L., Swings J. and Kersters K. 1991. Grouping of *Xanthomonas campestris* pathovars by SDS-PAGE of proteins. J. Gen. Microbiol. 137:1677-1687

Voronkevich I. V. and Shmanenkova T. N. 1968. Biological role of extracellular polysaccharides of *Xanthomonas phaseoli* var. *sojense* the casual agent of soybean bacteriosis. In, Swings, J. G. and E. L. Civerolo. 1993. Xanthomonas. Chapman & Hall. London

Widjaja R., Suwanto A. and Tjahjono B. 1999. Genome size and macrorestriction map of *Xanthomonas axonopodis* pv. *glycines* YR32 chromosome. FEMS Microbiol. Lett. 175(1): 59-68

Appendix

Data and ANOVA Tables

Table 1. Pustule development on a susceptible variety of soybean (PI520.733) inoculated with cells of *X. axonopodis* pv. *glycines* UIUC-1 grown on solid and liquid media

Number of Pustules		
Plant	Agar	Broth
1	50.5	19
2	13	30
3	24	25.5
4	47.5	15.5
5	78.5	22
6	23	38.5
7	30.5	39.5
8	16	6
9	31.5	32.5
10	38.5	23
11	74.5	4.5
12	49	95.5
13	53.5	7.5
14	81	7
15	63	53.5
16	54.5	23.5
X ± SE	46 ± 5	28 ± 6

ANOVA Table based on data from Table 1.

Model Terms	Df	Sum of squares	Mean squares	F	P
Replications	1	14671.266	14671.266	12.297	0.001
Agar-broth	1	5094.391	5094.391	4.270	0.043
Reps x agar-broth	1	735.766	735.766	0.617	0.435
Error	60	71583.813	1193.064		

Table 2. Average number of pustules on a susceptible variety of soybean (PI 520.733)
inoculated with cells of *X. axonopodis* pv. g*lycines* UIUC-1 grown on potato dextrose
agar (PDB), soytone peptone broth (SPB) and undefined glucose medium (UGM)

	Number of Pustules		
Plant	PDB	SPB	UGM
1	60	63	59
2	78	65	111
3	92	12	127
4	52	51	107
5	20	0	117
6	56	34	0
7	88	13	166
8	26	22	58
9	92	36	0
10	10	27	112
11	78	20	12
12	66	33	18
13	77	24	92
14	68	11	9
15	33	106	42
16	44	40	94
X ± SE	59 ± 7	35 ± 7	70 ± 13

ANOVA Table based on the data from Table 2

Model Terms	Df	Sum of squares	Mean squares	F	P
Replications	1	9087.042	9087.042	8.496	0.004
Media	2	22958.687	11479.344	10.733	0.000
Reps x Media	2	6511.021	3255.510	3.044	0.053
Error	90	96261.750	1069.575		

Table 3. Average number of pustules on a susceptible variety of soybean (PI 520.733) inoculated with cells of *X. axonopodis* pv. *glycines* UIUC-1 harvested at different phases of growth (4 h – lag phase, 8 h – mid-log phase, and 12 h – late-log phase)

	Number of Pustules		
Plant	4 h	8 h	12 h
1	29	31	48
2	43	53	13
3	21	72	39
4	53	42	27
5	32	21	74
6	11	65	33
7	30	78	77
8	33	14	15
9	51	62	0
10	23	20	36
11	35	39	51
12	11	118	34
13	27	39	52
14	2	40	77
15	22	57	19
16	2	38	66
X ± SE	27 ± 4	49 ± 7	41 ± 6

ANOVA Table based on data from Table 3

Model Terms	Df	Sum of squares	Mean squares	F	P
Replications	1	7722.094	7722.094	7.946	0.006
Time	2	8524.000	4262.000	4.385	0.015
Reps x Time	2	1336.750	668.375	0.688	0.505
Error	90	87465.813	971.842		

Table 4. Average number of pustules on a susceptible variety of soybean (PI 520.733) inoculated with cells of different isolates of *X. axonopodis* pv. *glycines*

Plant	Number of Pustules			
	UIUC-1	ATCC	UIUC-2	UF
1	108.5	39.5	18.5	21
2	103	25.5	62	29
3	102.5	45.5	34.5	12
4	76	74.5	35	69.5
5	27	4.5	48	39.5
6	52.5	26	10.5	8.5
7	60	30	34	22.5
8	133	39	38	56
9	54	83	31.5	37.5
10	36	52.5	30.5	28
11	66.5	44	14.5	57.5
12	48.5	28.5	15.5	55
13	70.5	41.5	27	7.5
14	61	22.5	17.5	37.5
15	27.5	19.5	30	26
16	136	18	21.5	37
X ± SE	73 ± 9	37 ± 5	29 ± 3	34 ± 5

ANOVA Table based on data from Table 4

Model Terms	df	Sum of squares	Mean squares	F	P
Replications	1	1785.031	1785.031	1.462	0.229
Isolates	3	37853.781	12617.927	10.335	0.000
Reps x Isolates	3	7378.656	2459.552	2.015	0.116
Error	120	146501.500	1220.846		

Table 5. Data from metabolic fingerprinting - Growth measured at 750 nm for all

bacteria isolates

Subtrates	UIUC-1	ATCC	UIUC-2	UF	Xcc	P.f.
Alpha-Cyclodextrin	-	+	+	-	-	-
Dextrin	+	+	+	+	+	-
Glycogen	+	+	+	+	+	-
Tween 40	+	+	+	+	+	+
Tween 80	-	-	+	-	-	+
N-acetyl-D-Galactosamine	+	+	+	+	+	+
N-acetyl-D-Glucosamine	+	+	+	+	+	+
Adonitol	+	-	-	-	+	-
L-Arabinose	-	-	+	-	+	-
D-Arbitol	-	-	+	-	+	-
D-Cellubiose	+	+	+	+	+	-
i-Erythriol	-	-	+	-	+	-
D-Fructose	+	+	+	+	+	+
L-Fucose	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+
Gentiobiose	+	+	+	+	+	-
Alpha-D-Glucose	+	+	+	+	+	+
m-Inositol	-	+	+	-	+	-
Alpha-D-lactose	+	+	+	+	+	-
Lactulose	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
D-Mannitol	-	+	+	+	+	-
D-Mannose	+	+	+	+	+	+
D-Melibiose	+	+	+	+	+	-
Beta--Methyl-D-Glucoside	-	+	+	+	-	-
D-Psicose	+	+	+	+	+	-
D-Raffinose	+	+	+	+	+	-
l-Rhamnose	+	+	+	+	+	-
D-Sorbitol	+	+	+	+	+	-
Sucrose	+	+	+	+	+	+
D-Trehalose	+	+	+	+	+	-
Turanose	+	+	+	+	+	-
Xylitol	-	-	+	-	-	-
Pyruvic Acid Methyl Ester	+	+	+	+	+	+
Succinic Acid Mono-Methyl	+	+	+	+	+	+
Acetic acid	-	-	+	+	+	+
Cis-Aconitic Acid	+	+	+	+	+	+
Citric Acid	+	+	+	+	+	+
Formic acid	+	-	+	+	+	+
D-Galactonic Acid Lactone	-	+	+	+	+	+
D-Galacturonic Acid	+	+	+	+	+	+
D-Gluconic acid	-	+	+	+	+	+
D-Glucosaminic Acid	+	+	+	+	+	-
D-Glucuronic acid	+	+	+	+	+	+
Alpha-Hydroxybutyric Acid	+	+	+	+	+	-
Beta-Hydroxybutyric Acid	+	+	+	+	+	+
Gamma-Hydroxybutyric Acid	-	-	-	+	-	-
Para-Hydroxy-phenylacetic	-	-	+	-	-	-
Itaconic Acid	-	+	+	+	+	+
Alpha-Ketobutyric Acid	-	+	+	+	-	-

57

	UIUC-1	UIUC-2	ATCC	UF	Xcc	P.f.
Alpha-Ketoglutaric Acid	+	+	+	+	+	+
Alpha-Ketovaleric Acid	-	-	+	-	-	-
D,L-Lactic acid	+	+	+	+	+	+
Malonic acid	+	+	+	+	+	+
Propioinic Acid	+	+	+	+	+	+
Quinic Acid	-	-	+	+	+	+
D-Saccharic Acid	+	-	+	+	+	+
Sebacic Acid	-	-	+	-	-	-
Succinic Acid	+	+	+	+	+	+
Bromosuccinic Acid	+	+	+	+	+	+
Succinamic Acid	+	+	+	+	+	-
Glucuronamide	+	+	+	-	-	+
L-Alaninamide	+	+	+	+	+	+
D-Alanine	+	+	+	+	-	+
L-Alanine	+	+	+	+	+	+
L-Alanyl-Glycine	+	+	+	+	+	+
L-Asparagine	+	+	+	+	+	+
L-Aspartic Acid	+	+	+	+	+	+
L-Glutamic Acid	+	+	+	+	+	+
Glycyl-L-Aspartic Acid	+	+	+	+	+	-
Glycyl-L-Glutamic Acid	+	+	+	+	+	+
L-Histidine	+	+	+	+	+	+
Hydroxy-L-Proline	+	+	+	+	+	+
L-Leucine	-	+	+	+	-	+
L-Ornithine	-	+	+	+	+	+
L-Phenylalanine	-	+	+	+	-	+
L-Proline	+	+	+	+	+	+
L-Pyroglutamic Acid	-	+	+	+	-	+
D-Serine	+	-	+	-	-	-
L-Serine	+	+	+	+	+	+
L-Threonine	-	+	+	+	+	+
D,L-Carnitine	+	+	+	+	-	+
Gamma-Aminobutyric Acid	-	-	+	-	-	+
Urocanic Acid	-	+	+	+	+	-
Inosine	-	+	+	+	+	+
Uridine	-	-	+	+	-	+
Thymidine	-	-	+	-	-	-
Phenylethyl-amine	-	-	+	-	-	+
Putrescine	-	-	+	+	-	+
2-Aminoethanol	-	-	+	-	-	+
2,3-Butanediol	-	+	+	+	+	-
Glycerol	+	+	-	+	-	+
D,L,alpha-Glycerol Phosphate	-	+	+	+	+	-
Alpha-D-Glucose-1-Phosphate	-	+	+	-	+	+
D-Glucose-6-Phosphate	-	+	-	+	+	-

+ indicates growth, - indicates no growth

UIUC-1, *X. axonopodis* pv. g*lycines* UIUC-1; UIUC-2, *X. axonopodis* pv. g*lycines*

UIUC-2; ATCC, American type culture collection 17915; UF, University of Florida;

Xcc, *X. campestris* pv. *campestris*; P.f., *Pseudomonas fluorescens*

Vita

Lopa Goradia was born 16 May 1977 in Mumbai, India. She received a Bachelor of Science degree in Microbiology from the University of Mumbai in 1998. She entered Eastern Illinois University for graduate studies and received a Master of Science in Biological Sciences under the direction of Dr. Steven Daniel. She is looking for a prospective job in her field.