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SYSTEMIC ACQUIRED RESISTANCE OF COMMON BEAN TO XCP AND PARTIAL CHARACTERIZATION OF BACTERIA ISOLATED FROM DISEASED AZUKI BEAN PLANTS.

By

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ABSTRACT

SYSTEMIC ACQUIRED RESISTANCE OF COMMON BEAN TO XCP AND PARTIALCHARACTERIZATION OF BACTERIA ISOLATED FROM AZUKI BEAN PLANTS

By Seriba Ousmane Katile

Two cultivars (Mayflower and Midland) of common bean (*Phaseolus vulgaris)*, when treated with 2,6 dichloroisonicotinic acid (INA) or inoculated with a low concentration of bacteria (*X. campestris* pv. *phaseoli*) exibited a resistant reaction when challenge inoculated with a high concentration of *Xanthomonas campestris* pv. *phaseoli*. In greenhouse experiments, INA caused a reduction in disease infection and severity on cultivars on both cultivars. The systemic resistance was accompanied by increased peroxidase activity. In field experiments, there was reduced infection on both cultivars and an increase of yield on the moderately resistant cultivar Mayflower.

Several bacteria were isolated from Azuki bean plants, seeds, and seedlings and tested for their characteristics and pathogenicity on common bean in greenhouse experiments. It appeared that some of the strains were pathogenic to common bean when they were inoculated on several cultivars. The pathogenic strains were tentatively identified as *Curtobacterium flaccumfasciens* and *Pseudomonas syringae*.

‘ To my children Moussa, Adama, Aissata,

and my late father and mother’

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PART I : SYSTEMIC ACQUIRED RESISTANCE TO COMMON BEAN TO XCP

I. INTRODUCTION.

Dry beans are susceptible to several disease causing bacteria. The three major bacterial diseases are common bacterial blight, halo blight and bacterial brown spot. The bacterial pathogens are *Xanthomonas campestris* pv. *phaseoli*, *Pseudomonas syringae* pv. *phaseolicola* and *Pseudomonas syringae* pv. *syringae*, respectively. Among these diseases, common bacterial blight is the most important and difficult to control because there is no commercially available resistance, and chemical control is not reliable.

Symptoms of common blight on leaves initially appear as water soaked spots that enlarge gradually, become flaccid and then necrotic. Lesions can be found throughout the leaf including the margins. In severe infections dead leaves may remain attached to the plant at maturity. Bacteria exude through stomata, thus providing inoculum for secondary infections. Pod lesions are generally circular, slightly sunken and dark red-brown.

Lesions are more abundant and severe on older leaves, but younger leaves can be affected. The disease affects plant vigor, reduces yield, and can affect seed quality. When the seed is internally contaminated, it is not possible to eradicate bacteria by seed treatment. During extended periods of warm, humid weather, the disease can be highly destructive causing loss in both quantity and quality of seed. Infected seeds are shriveled and exhibit poor germination and vigor. When plants are severely infected, the entire plant can die (Hall, 1991).

The bacterial pathogens causing common blight are *X. phaseoli* (Smith) Dawson and its fuscous variant *X. phaseoli* var. *fuscans* (Burkholder) Starr and Burkholder, which produces a brown pigment in culture. Both are now recognized as *Xanthomonas campestris* pv. *phaseoli* (Smith) Dye. It is a gram negative, straight rod, aerobic bacterium that is motile by a polar flagellum. The bacterium produces a yellow non-water soluble carotenoid pigment (xanthomonadin) and mucoid growth on nutrient glucose agar and YDC (yeast extract dextrose $CaCO_3$). Common blight of bean can develop from several inoculum sources but external and internal seed contamination are by far the most important means of survival for *Xanthomonas campestris* pv. *phaseoli*. The pathogen may overwinter in plant debris for at least a year and longer in infected seed (Saettler, 1989).

The primary methods of disease control include the use of pathogen-free seed and crop rotation. Chemical controls are not effective and all commercial cultivars of edible beans are susceptible to *X. campestris* pv. *phaseoli*. In the absence of traditional chemical and resistance strategies, an alternate method to reduce the disease is needed. Among the techniques developed recently, systemic induced resistance may be an option.

Some plants species may react to pathogen infection by the induction of a long-lasting broad - spectrum systemic resistance to subsequent infections by the same pathogen or even other pathogens. The phenomenon has been known for many years and has several names such as physiological acquired immunity (Chester 1933), induced resistance or systemic acquired resistance (Ross 1961b).

General overview of induced resistance:

Systemic acquired resistance is a broad, physiological immunity that result from infection with a necrotic pathogen (Kessmann et al 1994). In addition, certain natural or synthetic chemical compounds can trigger similar plant responses (Kessmann *et al*, 1994). Many plants develop an increased resistance against subsequent pathogen infection in uninfected tissues. This systemic acqured resistance can be effective against viruses, bacteria and fungi and is accompanied by the systemic expression of a group of genes called SAR genes (Kuc, 1982; Ward *et al*, 1991).

Most of the studies on systemic resistance have concentrated on tobacco (Ross 1961; Tuzun and Kuc; 1989) and cucurbits (Hammerschmidt *et al* 1976, Kuc, 1975), but some effort has focused on other crops including wheat (Ride, 1980), cabbage (Cook *et al* 1985), rice (Smith *et al*, 1991), millet (Kumar *et al* 1993), and sugar beet (Nielsen *et al* 1994).

Systemic induced resistance in plants is distinct from preexisting resistance mechanisms (i.e physiological barriers), protein cross- linking, and phytoalexin biosynthesis, the hypersensitive response and ethylene - induced physiological changes (Ryals *et al* 1994). Wounding or osmotic stress responses are not related to the induced systemic resistance (Ryals *et al* 1994).

The first step in the development of systemic acquired resistance (SAR) is the recognition by the plant of the pathogen infection. Compatible and incompatible interactions can lead to an induction of SAR; thus the pathogen needs not to induce a gene-for-gene resistance reaction (Kuc, 1982).

The induction of resistance usually coincides with the accumulation of pathogenesis-related (PR) proteins. PR proteins and salicylic acid (SA) accumulate in plant tissues following resistance inducing treatments that include inoculation with microorganism and treatment with some chemical compounds. Some examples of PR-proteins are chitinases (PR-3 group) and β-1, 3 glucanases (PR-2 group) which possess antifungal activity and may play an active role in disease resistance. Salicylic acid and 2, 6 - dichloroisonicotinic acid (INA) are two known chemical inducers of resistance (Nielsen *et al,* 1994). SA has induced both local resistance to TMV and the accumulation of PR proteins (White, 1979). There is evidence to suggest SA be an endogenous signal mediating disease resistance and an exogenous inducer of PR protein accumulation (White, 1979). 2,6 dichloroisonicotinic acid (INA) induces local and systemic resistance to pathogens in a number of plants (Kuc 1982).

Bean induced resistance:

The systemic induction of resistance on beans was first reported by Sutton (1979). The pre-inoculation of unifoliate leaves with spore suspensions of *Colletotrichum lindemuthianum* resulted in less severe symptom development in the first trifoliate leaves compared with control plants when those leaves were challenged with the same pathogen 7 or 12 days later (Sutton 1979). Green bean plants were protected against anthracnose caused by *Colletotrichum lindemuthianum* when the hypocotyls of bean cultivars resistant to some but not all the races of the fungus were inoculated, with nonpathogenic races of the organism (Kuc, 1982). Bean plants also developed resistance against pathogens after a primary infection by the same pathogen or other pathogens, or exposure to certain

chemicals (INA) that helped them to build up their defense system against *P.syringae* pv. *phaseolicola* (Dann and Deverall 1995).

Because conventional disease management strategies to control common bacterial blight of bean such as use of clean seed, rotation, burial of crop debris by plowing, chemicals and resistance are either not very effective or available, the research in this thesis addressed the possibility of using SAR as a disease management tool. The objectives of this study were:

1 - to study the effectiveness of systemic acquired resistance of dry beans to bacterial blight caused by *Xanthomonas campestris* pv. *phaseoli* in field and greenhouse experiments.

2 - to analyze the effect of inoculating plants with INA or bacteria on PR- proteins and peroxidase activities.

II. LITERATURE REVIEW:

Historical perspectives of Systemic Induced Resistance.

Systemic induced resistance or acquired systemic resistance has been recognized for many years by naturalists and scientists (Ryals 1994). The natural phenomenon of resistance development in response to pathogen infection was first recognized in 1901 by Ray & Beauverie, who worked with *Botryris cinera* on *Begonia*. Chester (1933) reviewed 200 publications describing the phenomenon he termed physiological acquired immunity. During the 30 years following Chester 's review, many papers were published but most of them were descriptive studies extending the earlier observations.

The first systematic study on systemic acquired resistance (SAR) was published by Ross in 1961 where he used TNV (Tobacco Necrosis Virus) which causes local lesions on *Nicotiana tabacum*, and demonstrated that infections of TNV were restricted by a prior infection of the same virus. Ross established the validity of plant immunization against virus diseases on tobacco (*Nicotiana tabacum*), bean *(Phaseolus vulgaris)*, and cowpea (*Vigna unguiculata*) using viruses as the inducing agents (Ross 1964, 1966). Plant immunization was subsequently expanded to include many hosts and viruses, bacteria, fungi, cellular components of infectious agents and chemicals (Kuc 1976, 1981, a, b; Kuc and Caruso, 1977; Kuc and Richmond, 1977; McIntyre *et al*, 1981). The hypersensitive reaction (HR) leading to systemic acquired resistance was first characterized in Sweet William plants infected with "carnation mosaic virus" (Gilpatrik and Weintraub 1952). The accumulation of groups of extracellular proteins called PR-

proteins (Pathogenesis related proteins) was correlated with the onset of SAR (van Loon 1982). White (1979) demonstrated that salicylic acid (SA) and certain benzoic acid (BA) derivatives induced both resistance and accumulation of PR- proteins. As a result, SA was considered as a possible endogenous signal (Van Loon and Antoniw, 1982).

Studies on cucumber and tobacco indicated that the lignification of the cell walls (Hammerschmidt et *al*, 1982) or the induction of hydrolytic enzymes (Boller, 1987) and other PR-proteins (Van Loon, 1985) were components of the induced defense mechanism. Gottstein et *al* (1989) found that solutions of K_3PO_4, K_2HPO_4, Na_3PO_4 and Na_2PO_4 sprayed on the underside of the first and second true leaves of cucumber induced systemic resistance in leaves three and four against anthracnose caused by *Colletotrichum lagenarum*. The induction of systemic resistance by the use of chemicals was studied by Metraux *et al* in 1991. 2,6-dichloroisonicotinic acid or INA (CGA 41396) is a chemical compound formulated by Ciba-Geigy Ltd. with 25% active ingredient (a. i) that acts indirectly by simulating the mechanisms of resistance in the host plant (Metraux *et al* 1991). They hypothesized that after a rapid uptake and translocation to other parts of the plants INA acted directly with the defense reaction and not through the intermediate systemic signal.

In 1991, Ward *et al*, using a tobacco /TMV model system showed that steady-state mRNA levels from at least nine families of genes were coordinately induced in uninfected plants and they referred to these families collectively as SAR genes (Ward *et al* 1991). SA has been proposed as one signal leading to SAR because its concentration rises dramatically after pathogen infection (Malamy *et al*, 1990, Metraux *et al*. 1990).

However, experiments by Hammerschmidt *et al* (1982) suggests that SA may not be a systemic signal. According to Ryals *et al* (1994), SAR can be conceptually divided into two phases: an initiation and transient phase that includes all of the events leading to the establishment of resistance; and the maintenance phase describing the quasi steady-state resistance that results from initiation.

Nielsen *et al* (1994) induced resistance in sugar beet to *Cercospora beticola* only after repeated foliar applications of INA with concentrations ranging from 10-100 ppm, but higher concentrations caused toxicity (necrosis). Four treatments with 25 ppm of INA induced complete local and systemic resistance with no signs of phytotoxicity but fewer treatments caused a delay in symptom appearance. Injection of INA directly in soil or plants, soil drenching or immersion of roots into solutions of INA did not affect fungal growth (Nielsen *et al* 1994). Vernooij *et al* (1995) found that the synthetic chemical INA acts via the SAR signal transduction pathway. They showed that INA does not induce SA accumulation in *Arabidopsis* and that INA is effective in transgenic plants unable to accumulate SA. This suggested that INA induced the SAR signal transduction pathway by acting either at the same site or downstream of SA translation.

Hoffland *et al* (1995) showed that induction of resistance was possible in plants which did not accumulate PR-proteins suggesting that the accumulation of PR-proteins was not a prerequisite for the induction of systemic resistance.

A single application of INA at low concentration was sufficient to induce resistance against several fungal and bacterial pathogens on different species of plants (Metraux *et al* 1991). Inoculating millet *(Pennisetum glaucum)* seedlings with a low

concentration of zoospores, Singh *et al* (1993) induced systemic resistance to downy mildew of millet caused by *Sclerospora graminicola* after challenging with higher concentration of the same pathogen.

Induced resistance to bacteria:

Resistance was induced in number of plant species to bacterial diseases. These include *Pseudomonas solanacearum* on tobacco (Seiquira, 1984), *Erwinia carotovora* subsp. *carotovora* on tobacco (Palva *et al*, 1994), *Pseudomonas lacrymans* on cucumber (Kuc, 1982), *Pseudomonas syringae* pv. *phaseolicola* on bean (Dann and Deverall 1995), after a primary inoculation with microbial or chemical compounds. Infection of *Arabidopsis thaliana* with turnip crinkle virus (TCV) leaded to resistance to TCV or *Pseudomonas syringae* (Ukness *et al*, 1992). Active resistance to black rot caused by *Xanthomonas campestris pv campestris* was induced in cabbage by inoculation with *Xanthomonas campestris* pv. *carotae* (Cook and Robeson, 1985).

Proposed mechanisms of systemic acquired resistance.

Systemic resistance results from the release of endogenous compounds that are translocated from slowly necrotizing cells to other locations in the plants (sequeira, 1983) For a systemic signal to increase resistance to a pathogen or insect, it must be perceived by plant cells (Hammerschmidt *et al*, 1993). For chemical signals, this may involve binding to a receptor molecule in the plasma membrane (Ryan, 1992). The penetration of appresoria of *C. lindemuthianum* into immunized cucumber was markedly reduced

whereas the germination of conidia was unaffected (Hammerschmidt, 1980; Hammerschmidt and Kuc 1982; Jenns and Kuc, 1977; Jenns and Kuc 1980; Richmond *et al* 1979). Lignification that occurs rapidly after penetration was localized to the invaded cells and a few adjacent cells and was associated with an increase in peroxidase activity. As with immunization, a single lesion on the inducer leaf resulted in a statistically significant systemic increase in peroxidase activity (Hammerschmidt *et al*, 1980). The systemic increase in peroxidase was associated with markedly increased activity of several peroxidases (Hammerschmidt, 1980).

Several reports present strong evidence that lignification is a plant disease resistance mechanism, (Asada *et al*, 1979; Henderson and Friend, 1979; Pearce and Ride 1978, 1980; Ride 1980; Vance *et al*, 1980). Lignification can restrict development of pathogens by several possible mechanisms: increasing the mechanical resistance of the host cell wall, reducing the susceptibility of the host cell wall to degradation by extracellular enzymes, restricting the diffusion of pathotoxins and nutrients, inhibiting the growth of pathogens by the action of toxic lignin precursors (Ride, 1980)

Signals involved in systemic resistance:

A number of chemical and non-chemical signals are involved in the induction of systemic defense response of plant against pathogens (Enyedi et al 1992, Malamy and Klessig, (1992). These include salicylic acid (SA), oligogalacturonides, the lipids derived signals, peptides, abscisic acid, and non chemical signals (Hammerschmidt, 1993). 2-6 dichloroisonicotinic acid (INA) is a chemical compound that induces resistance in plants

(Metraux, 1991). These compounds may affect different points in the signal transduction pathway.

Salicylic acid (2-hydroxybenzoic acid):

Salicylic acid plays a central role in SAR signal transduction after pathogen infection and is an exogenous inducer of PR-proteins accumulations and resistance (Kessmann *et al*, 1994). In higher plants, SA has been proposed to be synthesized from *trans*-cinnamic acid to SA, via the intermediates *ortho*-coumaric acid or BA (Ward *et al* 1991). Such pathways provide a link between pathogen induction of phenyl propanoid biosynthesis and SAR signal production (Ward *et al* 1991). SA is required for SAR but it is not the translocated signal molcule (Kessmann et al 1994)

SA plays a central role in SAR-signal transduction after pathogen infection and is as an exogenous inducer of PR-proteins accumulation and resistance. The mechanism by which SA induced gene expression is unknown, but a study by Chen *et al*, (993) speculated that SA may be mediated by catalase inhibition.

Chemical inducers of resistance:

2-6 dichloroisonicotinic acid.

2-6 dichloronicotinic acid (CGA 41396) (fig. 1) and its methyl ester (CGA 41397), both referred to as INA, induce systemic resistance in plants (Metraux *et al* 1991). These compounds provide good protection against fungal and bacterial pathogens on cucumber, rice and other crops in greenhouse and field experiments (Kessman *et al*

1994). The compounds have no significant *in vitro* activity and are not converted into antimicrobial metabolites (Kessman *et al* 1994). INA induces β −1, 3-glucanases, chitinases and 6-phosphoglucanate - hydrogenase (6-PGD) in tobacco, whereas phenylalanine ammonia-lyase (PAL), acidic proteases, peroxidases and phenylphenoloxidase were not affected by this compound (Kessman *et al* 1994). The enzymes not affected by INA are typically observed in a local defense response associated with the hypersensitive response (HR). INA apparently mimics the biological induction of systemic disease resistance without affecting responses linked with local necrosis. INA and its analogs sensitized plant tissue to respond to attempted infection with additional defense reactions faster than untreated plants, in addition to the induction of SAR genes and enzymes (Kauss *et al* 1992).

Kauss *et al* (1992) showed that parsley cell cultures pre-treated with INA produced various phenolpropanoid-derived metabolites much more rapidly following treatment with fungal elicitor than did the control. In rice, INA induced a pronounced increase in lipoxygenase activity within two days of application, whereas an inactive analog had no inducing effect (Smith and Metraux, 1991). The isonicotinic acid derivative N-cyanomethyl-2-chloroisonicotinade induced a high level of activity against the rice blast pathogens (Seguchi *et al* 1992). Levels of lipoxygenases increased as did general lipid metabolisms and peroxidases in chemically treated and blast-inoculated rice leaves compared to inoculated controls. These results may indicate that other defensive mechanisms are involved in the induction of SAR, besides those already characterized.

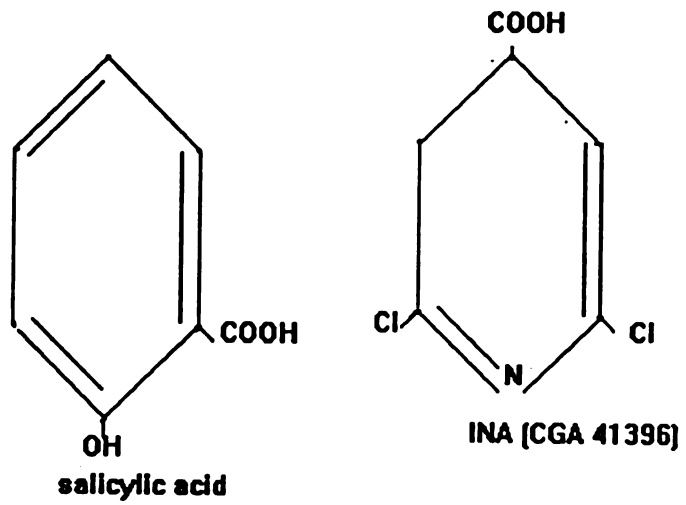


Fig. 1: Chemicals structure of Salicylic acid and INA (CGA 41996)

Peroxidase and chitinase activities.

Resistance in plants is associated with inducible compounds that may function in defense against disease: phytoalexins, peroxidases, lignin, hydroxyproline-rich glycoproteins (extensins) and pathogenesis related proteins (Hammerschmidt, 1982; Boller, 1987; van Loon, 1985). Increased peroxidase activity, enhanced lignification and extensin deposition are associated with induced systemic resistance in cucumber against *Colletotrichum lagenarium* (Hammerschmidt *et al,* 1982; Dalisay and Kuc ,1995). PR-proteins accumulation follows the induction of the systemic resistance (Smith and Hammerschmidt, 1988). The induction of chitinase activity occurred in different plant species in response to infection and treatment with fungal cell wall preparations (Dalisay, and Kuc, 1995). The observations that chitinase has inhibitory activity in *vitro* and apparently in *vivo* against some chitin-containing fungi but not those lacking chitin,

suggested a direct role of chitinase in plant defense.

Pathogenesis-related (PR) proteins:

Systemic induced resistance by pathogens or chemical agents is associated with a concomitant accumulation of pathogenesis-related (PR) proteins. Currently, five families of PR proteins have been classified (Carr and Klessig 1989). Within each family, members of the so-called class 1 proteins are generally located in the vacuole, whereas classes 2 and 3 are acidic extracellular proteins. The PR-1 family of acidic extracellular proteins have unknown function, but were the most abundant PR proteins in tobacco (Payne *et al* 1988b). They include PR-1a, PR- 1b and PR-1c. The PR-2 family of proteins includes class 1 basic vacuolar proteins, class 2 and 3 proteins, and acidic extracellular proteins with β-1,3 endoglucanase activity (Kauffman *et al* ., 1987, Ward *et al* 1991). The PR-3 family includes class 1 basic proteins and class 2 acidic extracellular proteins and endochitinase activity (Legrand *et al*., 1987; Payne *et al* 1990; Heitz *et al* 1994). PR-4 family includes acidic extracellular proteins with unknown function (Friedrich *et al* 1991). PR-5 family has class 1 basic proteins and class 2 acidic extracellular protein homologous to thaumatin (Payne *et al* 1988). Evidence is accumulating that PR proteins possess direct antifungal activity in *vitro* but their biological role in *vivo* is unclear. Working with *Pseudomonas fluorescens* as an inducer of resistance in the radish against *Fusarium oxysporium* f.s. *raphani,* Hoffland *et al (*1995) demonstrated that accumulation of PR proteins was not a prerequisite for the expression of induced systemic resistance.

Common bean-pathogen interaction.

The invasion of plant tissue by foliar bacterial pathogens generally occurs through natural openings, e.g., stomata, hydatodes or nectaries, when there is a water congestion, such as following a rainfall or heavy dew or under high humidity (Panopoulos and Schroth 1974). The bacteria then invade intercellular spaces, causing a gradual dissolution of the middle lamella (Panopoulos and Schroth !974). One of the most conspiscious effects of microorganisms on plant cell walls is enzymatic degradation (Walton 1994). Number of cell wall dergrading enzymes have been reported from plant pathogen; these include cellulase, pectinase, cutinase , xylase and protease (Walton 1994).

The most common simple interpretation of gene for gene system involving bacteria and fungi and for which there is direct evidence in a bacteria system is that the gene for avirulence controls the production of an elicitor, the recognition of which is controlled by the gene for resistance in the plant (Heath, 1991)

Colletotrichum lindemuthianum penetrates common bean by exerxing mechanical pressure but there is evidence that the action of cutinases enzymes is also required for successful penetartion of this pathogen (O'Connell *et al* 1985). Subsequent penetration of the epidermal cell appears to be mediated by enzymatic degradation and mechanical pressure (O'Connell *et al* 1985).

Common bean may respond to bacterial infection by different means and bacteria may also use different strategies to penetrate bean plant tissues. The role of gene-for-gene interaction may be determinant in some bacterial diseases especially halo blight.

Incompatible reactions lead to necrosis and death of the surrounding cells, and compatible reactions lead to disease development.

As described in the introduction there is no commercially available resistance to common bacterial blight *(Xanthomonas campestris* pv. *phaseoli*) of edible bean, and chemical controls have not been effective. The previous research on SAR suggested that this may be a practical approach to reduce the severe economic losses to this disease expected in Michigan. The objectives were:

1- to study the effectiveness of systemic acquired resistance in dry beans to bacterial blight caused by *Xanthomonas campestris* pv. *phaseoli* in field and greenhouse experiments.

2- To analyze the effect of inoculating plants with INA or bacteria on PR-proteins and peroxidase activities.

III. MATERIALS AND METHODS

Plant materials:

Two commercial cultivars of common bean (*Phaseolus vulgaris* L.) were used in this study: Mayflower and Midland. These cultivars are not as commonly grown in Michigan as they were several years ago, but their reaction to *X. campestris* pv. *phaseoli* is distinct: Mayflower is moderately susceptible, Midland is highly susceptible. Both cultivars were used in field and greenhouse experiments. Seed was provided by Dr. J. Kelly, Department of Crop and Soil Science (MSU).

Bacterial isolates:

The bacterial isolate used both in the field and greenhouse study was a strain of *X. campestris* pv. *phaseoli* (Smith) Dye (DWF 151) isolated from infected leaf tissue and obtained from Dr. D.W. Fulbright (Department of Botany and Plant pathology, MSU). The bacterial cultures were grown on NBY (nutrient-broth yeast extract agar) medium. The composition of this medium was the following: nutrient broth, 8 g/l; yeast extract, 2 g/l; K_2HPO_4, 2 g/l; KH_2PO_4, 0.5 g/l; glucose, 2.5 g/l; Bacto-agar, 15 g/l. The medium was autoclaved for 20 minutes and poured in 100 mm x 10 mm plastic petri dishes. After cooling, a metal loop was sterilized with alcohol and flamed, and single colonies were streaked on the solidified medium. Plates were kept under fluorescent light to enhance growth. A day before inoculation of plants, single colonies were picked with a sterile toothpick and dropped into 200 ml of nutrient broth / 500 ml Erlenmeyer flasks.

The flasks were placed in a controlled environment incubator shaker (New Brunswick Scientific) incubator shaker at 30°C and the bacteria were grown overnight. The bacterial solution was centrifugated at $1x10^4$ g in an IEC Centra-4B centrifuge and the pellet collected. The pellet was diluted in sterile water and a Beckman DB-G grating spectrophotometer was used to measure the optical density of the solution which was adjusted to an $O.D._{600} = 0.05$ for the inducing inoculation and $OD_{600} = 0.6$ for the challenge inoculation.

Greenhouse experiments:

Commercial navy bean cultivars Midland and Mayflower were grown in 15 cm clay pots filled with bacto-professional soil. For each treatment, 2 to 3 plants were used. Plants were watered daily and the temperature was maintained at 20 to 25° C during the day and 16 - 18° C during the night. Plants were fertilized once with a 10% water solution of urea to enhance growth. Plants received the primary SAR inducing inoculation two weeks after planting. The experiments were performed five times and the data for the 3 best experiments were collected.

Methodology for inoculation:

Primary resistance induction treatments: Three primary SAR inducing traetments were used to induce SAR in the dry bean cultivars: 1) injection of INA solution into the leaves; 2) soil drenches with a solution of INA; 3) inoculation of leaves with a low concentration of *X. campestris pv. phaseoli*. Primary leaves were injected with a 50-ppm water solution

of INA. Preparation of INA solution consisted of diluting 200 mg of the INA powder (25 % a.i) in 1 liter of distilled water (Hammerchmidt, personal communication). Approximatively 50 ul of INA solution were infiltrated in the lower surface at four to five locations using a 1 ml syringe without a needle. Leaf intercellular spaces were infiltrated by placing the leaf tissue between the syringe opening and the thumb and depressing the syringe plunger. For the soil drenches, the soil surface of each pot containing three plants was drenched with 100 ml of a 0.5 ppm solution of INA. For the bacterial primary SAR inducing inoculation, plants were inoculated with *X.campestris* pv. *phaseoli* strain DWF 151. The strain was grown on NBY (as described previously) for one week and single colonies collected and grown in a nutrient broth solution for 24 hours at 30^{0}C in a controlled environment incubator shaker (New Brunswick Scientific). Bacterial pellets were collected by centrifugation as described previously and diluted with sterile distilled water. Using small centrifuge tubes the pellet was diluted with distilled water and the optical density was adjusted an O.D. $_{600}$ = 0.05 using a spectrophotometer. Preliminary studies showed that an O.D. $_{600}$ = 0.05 corresponded to 10^5 cfu/ml^{-1}. The bacteria were infiltrated into the lower surface of the leaves using a 1 ml syringe, in the same manner as the INA solution.

A day prior to inoculation, the plants were placed in an air-conditioned mist chamber at 100% relative humidity and a temperature of 20^0 C. After inoculation, the plants were placed in plastic bags and kept in the mist chamber for 24 hours. The plastic bags were removed and plants then placed on benches in the greenhouse 5 to 7 days before the challenge inoculation. A day before the challenge inoculation, plants were

again placed in plastic bags in the mist chamber.

Challenge inoculation:

Plants were challenge inoculated with the same strain used in the primary inoculation *X. campestris* pv. *phaseoli* strain DWF 151 grown as described above, however the concentration of bacteria for the challenge inoculation was higher. The $O.D._{600}$ was 0.6 corresponding to (10^9 cfu/ml^{-1}). For the challenge inoculation the plants were sprayed to run off with strain DWF 151 strain using a hand field 550 ml sprayer, then re-bagged in plastic and kept in the mist chamber for an additional 2 to 3 days. The treatments were the following:

T_0: Control (no treatments).

T_1: INA inoculation only

T_2: INA soil drenches only.

T_3: Bacteria low concentration only - primary inoculation

T_4: Control + challenge with bacteria

T_5: INA inoculation + challenge with bacteria

T_6: INA soil drench + challenge with bacteria

T_7: Bacteria low cfu + challenge with bacteria.

Plants were evaluated 10-days after the challenge inoculation for typical lesions on leaves. The data collected included the percentage of infected plants, number of infected leaves, number of bacterial lesions per leaf or plant, disease severity, and the number of colony forming units (cfu / per leaf disc) at 1, 2, 4, 6, and 8, days after the challenge inoculation.

Multiple applications of INA.

To study the effect of increasing the number of INA applications on common blight infection, INA was sprayed one, two, or three times on both cultivars using a 250-ml hand sprayer. The first application of INA was 10 days after planting, the second application, 4 days after the first and the third application was made 4 days after the second. The data recorded included the total number of plants, the number of infected plant, the number of infected leaves, and disease severity. The severity was recorded from 0 to 4 where 0 = no symptoms, 1 = weak infection, 2 = moderate infection, 3 = severe infection, 4 = very severe infection. The results of multiple treatments were compared to the drench inoculation and pre-inoculation with bacteria. Phytotoxicity was recorded when it was observed.

Analysis of bacterial growth:

After the challenge inoculation, the disc culture method was used for analysis of bacterial growth (Fulbright personal communication). A one cm disc of the leaf was removed using a sterile cork borer for each treatment at different times after the challenge inoculation. For each treatment, discs were taken from a single leaf (one disc per treatment for each day of collect). The discs were surface sterilized with 20% bleach and homogenized in 0.9% saline solution. The homogenate was serially diluted and 0.1 ml of each dilution from each treatment was pipetted on NBY medium, and the number of colony forming units (cfu) determined. The experiments were performed three times.

Dry bean peroxidase induction:

The effect of resistance inducing treatments on peroxidase activity was tested by infiltrating the lower surface of cotyledon leaves (first unifoliate leaves) of 10 day old plants of Mayflower and Midland in the greenhouse with 500 ul of INA or 500 ul of bacterial suspension ($O.D_{.600}$ = 0.6 corresponding to 1 x 10^9 cfu/ml) at five to ten points. A 1-ml disposable syringe was used for infiltration as described previously. For soil drenches 100 ml of INA solution (0.5 ppm) was poured over the soil surface of each pot. Control plants received no treatments. Six days after treatments, the first and second trifoliate leaves were collected for peroxidase assays.

Treatment leaves were collected from the treated and control plants by cutting carefully the petiole about 5-10 mm from the base of the leaves. Three leaflets collected from different treatments were placed into separate 250 ml beakers and covered with 50 ml of an ice- water mixture. The beakers were placed into a desiccant chamber and vacuum applied for 15 to 20 minutes to infiltrate the leaves with water. The leaves were removed, blotted dry on a paper towel and then carefully rolled into 50 ml centrifuge tubes. The water in the intercellular space was collected by centrifugation at 1500 x g for 15 minutes in an IEC-Centra-4B Centrifuge. The intercellular fluid was transferred to 1.5 ml Eppendorf tubes and store tubes at -20^{0}C until analyzed for peroxidase activity. Two methods were used for this study, SDS-PAGE gel (Laemmli, 1970) and the native gel

Sample preparation for SDS-PAGE consisted of adding 10 ul of sample Buffer (see the appendix B) to 30 ul of each sample of intercellular fluid collected as described above. The samples were heated for five minute at 95^{0}C and the entire 40 ul was loaded

into single wells of a 12% SDS-PAGE gel (see appendix B). Electrophoresis was carried out according to Laemmli (1970) in a Bio-Rad Mini-Protean II system (Bio-Rad, Hercules, CA) at the recommended power of 200 volts for optimal resolution with minimal thermal band distortion. The current was approximatively l0 mA per gel and the running time was 45 minutes. The gel was stained in Coomassie brilliant blue R250 overnight and then destained in a solution of 50% methanol, 10% acetic acid and 40% distilled H_2O.

The native gel was run following the procedure described by Hammerschmidt (1990). Approximatively 50 ul of each sample of intercellular fluid was collected as described above, and mixed with 1 ul of blue dye (see appendix A). The sample was loaded in a 7.5 % Acrylamide native gel (see appendix A). The current was set to run at 50 volts overnight, approximatively 16 hours. The gel was stained in a solution containing 120 mg of 4-dichloro-1- naphthol, 40 ml methanol, 20 ml of 10 X-PBS pH 7.3, 0.38 ml of hydrogen peroxide and 140 ml of ddH_2O.

Field experiments:

During the summer of 1995, Mayflower and Midland were grown in the field to evaluate several INA treatments on the development of common blight. This experiment was performed to confirm the greenhouse results on the effect of INA on disease reduction on bean. The plants were grown on the Botany East Farm in a sandy soil in 75 cm rows. Each treatment plot consisted of four rows of plants, 3 m long. All of the rows in the treatment plot were treated, but only the center 2 rows were rated for disease

severity and yield. There was a single border row of plants between every four rows of treated plants. The treatments were replicated 3 times in a completely randomized design.

The date of planting was July 5, 1995 and a post emergence herbicide was applied on July 24, 1995. INA was sprayed up to 3 times using a one - gallon hand sprayer (GREENLAWN, model 010PEXG, Gilmour, PA). The INA solution was sprayed over the plant until small drops of solution remained on the surface of the top leaves. The first application of INA was July 27 (week 1), the second on August 4 (week 2) and the third on August 11 (week 3).

The concentration of INA was 50 ppm which was applied in 20 gallons of water per acre. Challenge inoculation with bacteria was on August 23, 1995. The concentration of bacteria was 10^7 cfu/ml^{-1} corresponding to a spectrophometer reading $O.D._{.600} = 0.1$ on the Beckman spectrophotometer. Challenge inoculation was four weeks after the first application of INA. The treatments were as the follows:

1: Control, no treatment.

2: INA, 1 spray at week 1 + challenge inoculation with bacteria.

3: INA, 2 sprays at weeks 1 and 2 + challenge inoculations with bacteria.

4: INA, 3 sprays at weeks 1, 2 and 3 + challenge with bacteria.

5: INA, 1 spray, at week 2, no challenge inoculation.

6 : bacteria inoculation only (no INA spray).

7: INA, 1 spray at week 3 + challenge with bacteria.

Disease incidence was measured by counting the number of individual lesions on leaves from the inoculated plants during the 2 weeks following the challenge inoculation.

The plot was harvested for seed yield on a dry weight basis on October 12. The data were analyzed by one way analysis of variance using MSTATC (MSU, Crop and Soil Science).

III. RESULTS.

GREENHOUSE EXPERIMENTS:

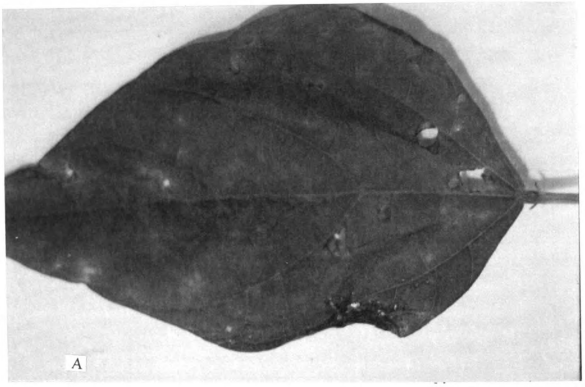
Effect of INA on common blight infection

Pre-treatment with 2-6 dichloroisonicotinoc acid (INA) reduced the rate of infection on cultivars Mayflower and Midland challenge inoculated with *Xanthomonas campestris* pv. *phaseoli* (Tables 1 and 2). Pre-treatment with INA by syringe injection or soil drenches provided about equal levels of control and were better than a pre-inoculation of cotyledons with *X. campestris* pv. *phaseoli.* Lesions were more prevalent on control plants challenge inoculated with *X. campestris* pv. *phaseoli* than on plants pre-treated with INA or bacteria (Fig 2A, 2B, and Fig 3C and 3D), and challenge inoculated with *X. campestris* pv. *phaseoli*. A primary inoculation with a low concentration of *X. campestris* pv.*phaseoli* was more effective on the cultivar Mayflower (Table 1) than the cultivar Midland (Table 2). The results indicated that a primary treatment with INA or *X. campestris* pv. *phaseoli* reduced the amount and severity of disease on both dry bean cultivars (Table 1 and 2). The differences between the cultivars Mayflower and Midland receiving a primary inoculation with *X. campestris* pv. *phaseoli* may have been due to the higher susceptibility of the cultivar Midland to this pathogen. The cultivar Mayflower was more resistant to common blight pathogen. These results tend to confirm field observations that Mayflower is moderately susceptible and Midland highly susceptible (Dr. J. Kelly, personal communication).

Fig 2 : **Resistant reaction on leaf of common bean cultivar Mayflower pre-treated with 2,6-dichloroisonicotininc acid and challenge inoculated with *Xanthomonas campestris* pv. *phaseoli* after 7 days.**

A. Control challenge inoculated with *X. campestris* pv. *phaseoli.*

B. INA pre-treated and challenge inoculated with *X.campestris* pv. *phaseoli.*


A

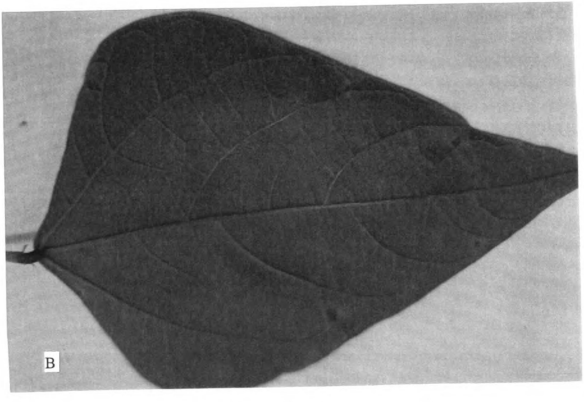
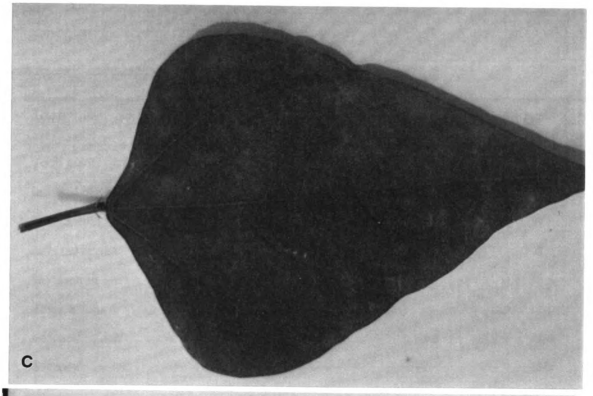

B

Fig. 3: Resistant reaction on leaf of common bean cultivar Mayflower pre-treated with 2,6 dichloroisonicotinic acid or bacteria and challenge inoculated with *Xanthomonas campestris* pv. *phaesoli* after 7 days.

C. INA drench and challenge inoculated with *X. campestris* pv. *phaseoli*

D. Primary and challenge inoculated with *X.campestris* pv.*phaseoli*.


C

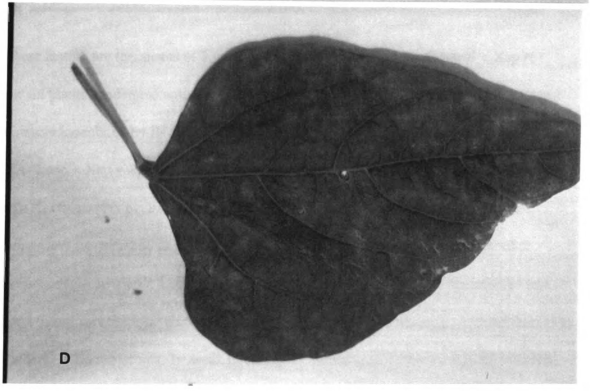

D

Table 1: Effect of pre-treating the common bean cultivar Mayflower with 2,6-dichloroisonicotinic acid on infection and disease severity after challenge inoculation with *Xanthomonas campestris* pv. *phaseoli*.

Treatments	Control+Xcp	INA inoc + Xcp	INA drench +Xcp	Xcp + Xcp
Total plants	2	3	2	2
Infected plants	2	3	1	2
Total leaves	32	47	30	39
Infected leaves	8	7	4	8
% infection	25	14.9	13.3	20.5
Total lesions	53	12	7	11
Lesions/ inf.leaf	6.6	1.7	1.8	1.4
Severity	4	1	2	3

These results are the means of 3 greenhouse experiments in 1995. Control + Xcp = control plants challenged with *X. campestris* pv. *phaseoli*; INA inoc + Xcp = plants primarily inoculated by INA injection and challenged with *X. campestris* pv. *phaseoli;* INA drenc + Xcp = Plants primarily inoculated with INA solution in soil and challenged with X. *campestris* pv. *phaseoli;* Xcp + Xcp = plants primarily inoculated with *X. Campestris* pv. *phaseoli* at low cfu / ml and challenged later with high cfu / ml of *X campestris* pv. *phaseoli.* Lesion/inf. Leaf = average number of lesions per infected leaf. Total lesion was the total number of lesions on all the leaves. Severity recorded from 0 to 4 where 0= no symptoms, 1= weak infection, 2= moderate infection, 3=severe infection, 4= very severe infection. Data were recorded 10 days after challenge inoculation.

Table 2: Effect of pre-treating the common bean cultivar Midland with 2,6-dichloroisonicotinic acid on infection and disease severity after challenge inoculation with *Xanthomonas campestris* pv. *phaseoli*.

Treatments	Control + Xcp	INA inoc + Xcp	INA drench + Xcp	Xcp + Xcp
Total plants	2	2	2	3
Infected plants	2	1	2	2
Total leaves	54	45	48	54
Infected leaves	11	3	6	9
% infection	20.4	6.7	12.5	16.7
Total lesions	42	13	14	32
Lesion/ inf.leaf	3.8	4.3	2.3	3.5
Severity	4	1	1	4

The results are the means of 3 greenhouse experiments in 1995. Control + *Xcp* = control plants challenged with *X campestris* pv *phaseoli*; INA inoc + *Xcp* = Plants primary inoculated by INA injection and challenged with *X. campestris* pv. *phaseoli*; INA drench + *Xcp* = Plants primary inoculated with INA solution in soil and challenged with *X.campestris* pv. *phaseoli*; *Xcp* + *Xcp* = Plant primary inoculated with *X. Campestris* pv *phaseoli* at low cfu/ml and challenged later with high cfu/ml of *X. campestris* pv.*phaseoli*. lesion/ inf.leaf = average number of lesions per infected leaf. Total lesion are the total number of lesions on all the leaves. Severity recorded from 0 to 4 where 0= no symptoms 1= weak infection; 2 = moderate infection; 3 = severe infection, 4= very severe infection. Data were recorded 10 days after challenge inoculation

Multiple applications of INA on the control of Xcp.

INA was sprayed onto plants 1, 2 or 3 times to evaluate the effect of multiple applications of INA on the control of common blight (Tables 3 and 4). It appeared that increasing the number of treatments with INA influenced infection by *X campestris pv. phaseoli.* On control plants inoculated with bacteria only, disease symptoms appeared on all leaves. Symptoms were severe on the top leaves of the control plants with wilted and dead leaves on the cultivar Mayflower. On the cultivar Midland, symptoms were even more severe on control plants inoculated only with *X. campestris* pv. *phaseoli.* Top leaves were dead and all the bottom leaves were necrotic. Both cultivars receiving a single primary treatment of INA (INA 1) and challenged with *X. campestris* pv. *phaseoli* had disease symptoms on the second and third trifoliate leaves but the top leaves were healthy. With two applications of INA, symptoms were visible only on the third trifoliate leaves after challenge inoculation on Mayflower, but on Midland, almost all the top leaves were severely wilted. With 3 applications of INA followed by a challenge with *X. campestris* pv. *phaseoli,* symptoms were generally localized on the fourth trifoliate leaves and all of the bottom leaves were healthy. Two and three applications of INA on Midland resulted in short plants with small leaves and symptoms were present only on fourth leaves. Curling of the top leaves was visible on all plants of the cultivar Midland receiving three sprays of INA.

Table 3. Effect of multiple applications of INA on infection and disease severity on cultivar Mayflower challenge inoculated with *Xanthomonas campestris* pv. *phaseoli*.

Treatments	Cont+Xcp	Ina1+Xcp	Ina2+Xcp	Ina3+Xcp	InaD+Xcp	Xcp+Xcp
Total plants	3	3	3	4	3	3
Infected plants	3	3	2	3	3	3
Total leaves	41	50	60	103	69	48
Infected leaves	20	9	10	6	14	17
% infection	48.8	18	16.7	5.8	20.3	35.4
Total lesions	47	11	14	9	39	50
Lesion/leaf	2.3	1.2	1.4	1.5	2.8	2.9
Severity	4	2	1	1	2	3

Note: These are the means of 4 greenhouse experiments in 1995 and 1996 cont+ Xcp = control inoculated with *X.campestris pv. phaseoli*, Ina1 + Xcp = one spray of INA + Challenge with Xcp, Ina2 + Xcp = Two sprays of INA and challenge with Xcp, Ina3 + Xcp = three sprays of INA + challenge with Xcp, Ina D + Xcp = INA in soil drench and challenge with *X.campestris pv phaseoli*. Xcp + Xcp : primary and challenge inoculation with *X.campestris pv phaseoli*. These data were recorded 10 days after challenge inoculation. Total lesions the total number of lesions on all the leaves of plants. Lesion/inf. leaf= average number of lesion per infected leaf.

Disease severity was recorded on a scale from 0 to 4 where 0 = no symptoms, 1= weak infection, 2 =moderate infection, 3 = severe infection, 4 = very severe infection.

Table 4: Effect of multiple applications of INA on infection and disease severity on the cultivar Midland challenge inoculated with *Xanthomonas campestris* pv. *phaseoli*.

Treatments	Cont+Xcp	Ina1+Xcp	Ina2+Xcp	Ina3+Xcp	InaD+Xcp	Xcp+Xcp
Total plants	3	2	3	4	3	3
infected plts	3	2	1	2	3	3
total leaves	53	56	44	72	69	58
infected leav.	25	9	4	7	23	22
% infection	47.2	16.0	9.0	9.7	33.3	37.9
Total lesions	58	18	9	8	45	61
lesion/ inf.plants	2.3	2	2.4	1.1	1.9	2.7
Severity.	4	1	1	1	2	3

These are the means of 4 greenhouse experiments in 1995 and 1996. Severity Cont+Xcp = control plant challenged with *X.campestris* pv. *phaseoli*. Ina 1 + Xcp = one spray of INA + challenge with Xcp, Ina 2 + Xcp = Two sprays of INA + challenge with Xcp, Ina 3 + Xcp = Three sprays of INA + challenge + Xcp; INA D + Xcp = INA in soil drench and challenge with Xcp; Xcp + Xcp = Primary and challenge inoculation with Xcp. These data were recorded 10 days after challenge inoculation. Total lesions are the total number of lesions on all the leaves. Lesion/inf. leaf = average number of lesions per infected leaf.

The disease severity was recorded from 0 to where 0 = no symptoms, 1 = weak infection, 2 = moderate infection, 3 = severe infection, 4 = very severe infection.

Analysis of bacterial growth

After the challenge inoculation, leaves of both cultivars Midland and Mayflower were collected from each treatment at 1, 2, 4, 6 and 8 days after the challenge inoculation with *X. campestris* pv. *phaseoli* to analyze the effect of INA on bacterial growth.

The bacterial population increased to high levels on control plants receiving only a challenge inoculation compared to plants pre-treated with INA or *X. campestris* pv. *phaseoli* and challenged inoculated with the same bacteria (Tables 5 and 6). On plants treated with INA, the population per ml increased at a slower rate than on the control plants (Tables 5 and 6). The population of Xcp from the cultivar Mayflower (Table 5) was generally lower than from Midland (Table 6) at each sampling period, and the total number of cfu's after 8 days was slightly less on Mayflower.

Table 5. The effect of 2,6 dichloroisonicotinic acid treatments on colony forming units of *X. campestris* pv. *phaseoli* from leaf discs of cultivar Mayflower after challenge inoculation.

	DAYS AFTER CHALLENGE INOCULATION				
Treatments (cfu)	1	2	4	6	8
	number of cfu/ml				
control + Xcp	1×10^4	2×10^4	7×10^5	5.5×10^6	7×10^5
INA 1 + Xcp	1.5×10^4	9×10^4	2×10^4	2×10^4	2×10^4
INA 2 + Xcp	9×10^3	6×10^3	1.2×10^4	1.2×10^4	1.2×10^4
INA 3 + Xcp	1×10^3	4×10^3	4×10^4	4×10^4	4×10^4
INA D + Xcp	10	7×10^3	1×10^5	1×10^5	1×10^5
Xcp+Xcp	5×10^2	2×10^2	2×10^2	3.5×10^5	5×10^5

Results are the means of 3 experiments.

Leaf discs were take on the same leaf for each treatment at different dates .

cfu = colony forming units

Table 6: Effect of 2,6 dichloroisonicotinic acid treatments on colony forming units of *X. campestris* pv. *phaseoli* from leaf discs of the cultivar Midland after challenge inoculation.

	DAYS AFTER CHALLENGE INOCULATION				
Treatments	1	2	4	6	8
	Number	of cfu / ml			
control +Xcp	1.2×10^5	1.1×10^4	9×10^5	1×10^6	7×10^6
INA1 + Xcp	2×10^4	8×10^4	2×10^5	2×10^5	2×10^5
INA2 + Xcp	4×10^3	1×10^4	0	1×10^4	1×10^4
INA3 + Xcp	2×10^4	0	0	0	2×10^5
INAD+ Xcp	2×10^4	6×10^4	1×10^4	1×10^4	1×10^5
Xcp + Xcp	0	6×10^3	5.5×10^6	5.5×10^6	5.5×10^6

The results are the means of 3 experiments.

Leaf discs were taken on the same leaf for each treatment at different dates.

For 2 and 3 applications of INA, some treatements have no cfu in the first day following the challenge inoculation.

Peroxidase activity:

The two cultivars of edible bean Mayflower and Midland were pre-treated with INA solution by injection, soil drench or bacterial solution by injection to study their effect on peroxidase activity. Extracts from intercellular fluid were used to analyze the effect of INA inoculation on peroxidase. Plants receiving a primary treatment with INA by injection and those inoculated with a low concentration of bacteria showed stronger protein bands (Figs 4 and 5). There was an increase in peroxidase activity in both cultivars Mayflower and Midland when treated plants were compared to control plants (Fig. 6). Bands appeared stronger than on control plants which showed a weak peroxidase activity in gel electrophoresis (Fig.6). These results suggested that increased protein accumulation and peroxidase activity occurs in bean plants treated with INA or bacteria solution.

Fig 4. SDS-polyacrylamide gel electrophoresis for proteins from the leaf of cultivar Mayflower (Coomassie blue stain).

Control = control plant (no pre-treatments) (no pre-treatment); INA inoc = plant pre-treated with INA by injection; INA drench = plant pre-treated with INA in soil drench; Xcp inoc = plant pre-treated with *X.campestris* pv. *phaeoli* in low concentration, INA + Xcp = plant pre-treated with INA by injection and challenge inoculated with *X.campestris* pv. *phaseoli.*

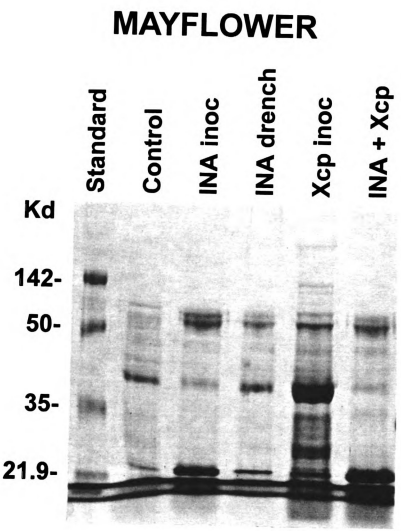

MAYFLOWER
Standard
Control
INA inoc
INA drench
Xcp inoc
INA + Xcp
Kd
142-
50-
35-
21.9-

Fig 5: SDS-polyacrylamide gel electrophoresis for protein from leaf of cultivar Midland (Coomassie blue stain).

Control = control plant (no pre-treatment); INA inoc = plant pre-treated with INA by injection; INA drench = plant pre-treated with INA in soil drench; Xcp inoc = plant pre-treated with *X.campestris* pv. *phaeoli* in low concentration, INA + Xcp = plant pre-treated with INA by injection and challenge inoculated with *X.campestris* pv. *phaseoli.*

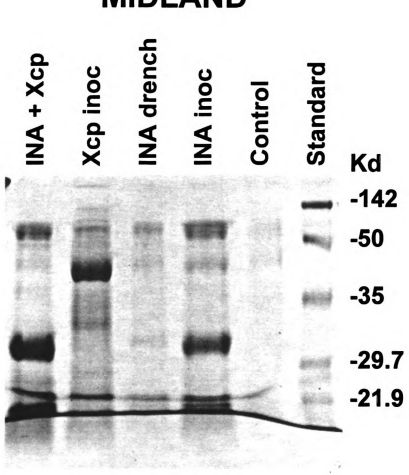
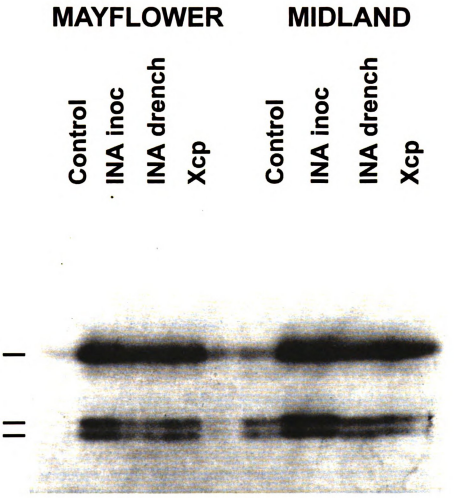

MIDLAND
INA + Xcp
Xcp inoc
INA drench
INA inoc
Control
Standard
Kd
-142
-50
-35
-29.7
-21.9

Fig 6: Native gel electrophoresis for peroxidase activity from the cultivars Mayflower and Midland. Control = control plant (no pre-treatments); INA inoc = plant treated with INA by injection; INA drench = plant treated with INA by soil drench; Xcp = plant treated with *Xantomonas campestris* pv. *phaseoli* in low concentration.


MAYFLOWER
MIDLAND
Control
INA inoc
INA drench
Xcp
Control
INA inoc
INA drench
Xcp
I
II

FIELD EXPERIMENTS.

Effect of INA on bacteria infection

The 1995 growing season was characterized by periods of drought during the month of August. It appeared that challenge inoculation of bean plants was affected by the drought since there was no supplemental irrigation. INA had different phytotoxic effects on the two cultivars Mayflower and Midland . While Mayflower did not have a noticeable response to INA, curling of leaves was clearly evident on the cultivar Midland especially after 3 applications of INA.

On the cultivar Mayflower, the application of INA significantly reduced the severity of infection, as determined by the average number of infected plants only after three primary treatments with INA and challenge inoculated with *X.campestris* pv. *phaseoli* (Table 7). Statistically, 1, 2 or 3 applications of INA significantly reduced the percentage of infected plants in the variety Midland compared to the control plants also challenged with *X.campestris* pv. *phaseoli*. A single late application of INA also reduced subsequent infection on Midland by *X. campestris* pv. *phaseoli*, but not on the cultivar Mayflower. Midland responded better than Mayflower to INA treatments when the number of infected plants was compared. However, overall disease severity was low even when the number control plants infected was as high as forty six percent (Midland table 7). One, two and three applications of INA on Midland significantly reduced the number of infected leaves but only three applications on Mayflower resulted in a significant reduction (Table 7). Mayflower appeared to be less susceptible than Mildland to infection by *X. campestris pv. phaseoli* (Tables 6 and 7)

Table 7. Effect of INA treatments on the number of infected plants on the bean cultivars Mayflower and Midland challenge inoculated with *X. campestris* pv.*phaseoli* in field experiments.

cultivars	Mayflower		Midland	
Treatments	% infection	% of control	% infection	% of control
Control (no treats)	24.5 ab	92.03	35.10 b	76.00
INA 1 + Xcp	14.11 abc	53.00	20.75 cd	44.95
INA 2 + Xcp	21.97 abc	82.53	9.46 e	20.56
INA 3 + Xcp	11.24 c	42.22	13.29 de	28.89
INA 1 late + Xcp	18.08 abc	67.91	24.26 c	52.62
INA only(no chall)	13.52 bc	50.78	21.18 cd	45.94
Control + Xcp	26.62 a	100.00	46.16 a	100
LSD	12.71		9.09	
CV.	40.16		40.8	

Note: Means followed by the same letters are not significantly different. Control plants did not receive any INA or challenge inoculation, INA 1 = 1 application of INA in week 1, INA2 = application on INA in week 1 and 2, INA 3 = application of INA in week 1, 2 and 3, INA 1 late = 1 application in week 3. All treatments were challenge inoculated 1 week after the last application of INA in treatment INA 3.

% of infection = percentage of plants infected by Xcp.

Effect of INA treatment on yield.

The field plot was harvested on October 12, 1995. Mayflower plots receiving 1, 2, and 3 applications of INA still had some plants with green leaves and stems while control plants not treated with INA were completely dry. On the cultivar Midland, plants from all treated plots were mature (i.e dry) but some curling of leaves was evident before harvest. There were no major differences in maturation between the different treatments in the cultivar Midland although 2 and 3 applications of INA did leave some green leaves in some plots. All the other plants were dried.

On Mayflower, all of the INA treatments significantly increased seed yields regardless of whether there was a challenge inoculation (Table 8), except for the single late application of INA followed by challenge inoculation with *X.campestris* pv. *phaseoli*. This was true for both wet and dry weights. Statistical analysis indicated no significant differences in wet or dry weight seed yields between different treatments on the cultivar Midland (Table 8).

Table 8: Effect of INA treatments on the bean cultivars Mayflower and Midland after challenge inoculation with *X. campestris* pv. *phaseoli* on seed yields.

Cultivar	Mayflower	Midland
Treatments	dry weight g/m2	dry weight g/m2
Control (no treats)	72.44 c	64.82 NS*
INA 1 + Xcp	85.11 a	56.64
INA 2 + Xcp	86.44 a	66.71
INA 3 + Xcp	76.44 b	55.57
INA 1 only (no chal)	88.77 a	70.53
INA (late) + Xcp	66.42 d	61.37
Control + Xcp	57.06 e	56.91
CV.	25.75	26.28

Average yields from the two central rows of each plot in three replications . INA 1+ Xcp= INA application in the first week and challenged 3 weeks later with Xcp; INA 2 + Xcp = INA application first and second weeks and challenged 2 weeks later with Xcp; INA 3+ Xcp = INA application in first, second, and third weeks and challenged one week later with Xcp; INA1 only (no chal) = INA application in the first week and no challenge with Xcp; INA (late) + Xcp= INA application in third week only and challenged on a week later with Xcp. NS*: no significant difference between treatments. These data are the results of one year experiment. Numbers followed by the same letter are not significantly different according to the Fisher Anova analysis. The dry weight was

DISCUSSION AND CONCLUSIONS:

The results from this study confirmed previous work on systemic resistance in edible beans. The observed reduction in disease severity caused by a foliar bacterial pathogen of bean following pre-treatment with a pathogen or chemical is consistent with the results of earlier work on bean by Sutton (1979); Cloud and Deverall (1987); (Dann and Deverall, 1995), and on other plants (Kuc, 1990; Hammerschmidt and Kuc , 1990; Metraux *et al*, 1991). Bean plants have shown resistance to many foliar diseases including anthracnose (Cloud and Deverall, 1987), rust (Dan and Deverall, 1995), and soilborne diseases (Kuc, 1990) after they were systemically induced. This was the first report on the induction of systemic acquired resistance in bean to the common blight pathogen *X. campestris* pv *phaseoli.*

The two culivars of common bean responded differentially to the induction of the systemic resistance. The cultivar Midland, highly susceptible to common blight was sensitive to INA treatments when the number of applications was increased by some visible signs on the leaves. The curling and reduced size of leaves were some of the signs on this cultivar in the greenhouse as well as in field experiments. On the cultivar Mayflower, there were no particular signs of phytotoxicity but maturity was delayed by INA application in the field experiments. INA applied as a soil drench appeared not to provide as good level of protection in bean against *X.campestris* pv. *phaseoli* on either of the two cultivars. Inoculation with a low concentration of bacterial suspension, although more effective on the reduction of disease infection than the control when they were

challenged was also not as effective in protecting the two cultivars as injecting INA into the leaves. The primary inoculation with bacteria lead to small lesions on the leaves that might have been a source of inoculum for other parts of the plants by the same pathogen.

INA treatment directly into the leaves may delay the infection of bean by *X. campestris* pv. *phaseoli*. The number of INA applications affected disease development by reducing the number of infected leaves, the number of lesions on the leaves and the severity of the disease. The first leaves adjacent to the INA treated leaves had better protection than upper leaves. This was evident by the presence of more lesions on the top leaves than on the bottom leaves of both cultivars when plants where inoculated two or three times with INA and challenge inoculated with *X. campestris* pv. *phaseoli*. Symptoms appeared earlier on control plants challenge inoculated with bacteria than on plants receiving INA treatments. Therefore, INA application may delay the infection of the bean plant by *X. campestris pv. phaseoli*. The method of pre-treatment can also affect the induction of resistance. Injections provided better induction than spray.

The number of colony forming units was not affected by INA or bacteria treatments during the first 3 days after the leaves were treated but by four days more colony forming units were present on control plant leaves. Plants receiving INA treatment had less cfu's on NBY medium than control or plants treated with bacteria in both cultivars. The cultivar Midland had a higher number of cfu's on control plants than on the cultivar Mayflower. The induction of resistance of bean plants was correlated with an increase in peroxidase activity for both cultivars of common bean. Both the native and polyacrylamide gels electrophoresis of plant intercellular space fluid showed that plants

treated with INA or bacteria had similar bands which were missing or weak on control plants (fig. 4, 5, and 6)

In the field experiments, the early application of INA resulted in a reduced percentage of infected leaves by *X.campestris* pv.*phaseoli* after the challenge inoculation. INA reduced infection of the plants challenge inoculated with bacteria below that of the control plants naturally infected by *X.campestris* pv. *phaseoli.* A late single application of INA was not as effective as a single early application. Two or three applications of INA reduced the amount of infection, but did cause some injury on the cultivar Midland.

The results of the field experiments confirmed those obtained from the greenhouse about the role of INA in the systemic induction of resistance in bean plants. INA provided good protection on both cultivars of bean against bacterial blight by reducing the number of infected leaves, the percentage leaf infection, disease severity and the number of bacterial cfu's on both cultivars in the greenhouse. In field experiments, INA reduced the percentage of infected plants on both cultivars and increased the seed yield on the cultivar Mayflower. Based on the results of percentage of leaf infection, disease severity, number of cfu's, and peroxidase activity in the greenhouse experiments and the percentage of infected plants in field experiments, it appeared that the susceptible cultivar Midland responded better to SAR than Mayflower. INA treatment may have contributed to the increase in yield in Mayflower, but on Midland, the phytotoxic effect observed on this cultivar (curling of leaves) could have reduced the yield because of its sensitivity to INA.

The potential impact of INA on bean production is not clear. Many diseases are responsible for the reduction of yield in different species of plants. The cultivar

Mayflower showed an increase in seed yields on plants inoculated with INA over the control, but on Midland, there was a decrease in yield in plants treated two to three times with INA. The results, although limited suggest a cultivar INA interaction that would require an evaluation of INA on every cultivars to determine if SAR was of potential benefit for specific cultivar. Future studies should include more cultivars of bean both in greenhouse and in field evaluations. Cultivar specificity has been shown to be important in the induction of SAR by bean plants and will have to be considered before INA can become a component of disease management practices.

The increase in peroxidase activity in greehouse experiments was a good indication of the systemic induction of resistance in bean plants. Increasing peroxidase activity followed INA treatments indicated that the defense system of bean responded to further infection of the plants by pathogens. In greehouse experiments, the decrease in lesion number in INA treated plants indicated a slow bacterial growth. This was confirmed by the low number of cfu's on INA treated plants. INA treatments also reduced the disease severity to a low level (generally less than 10 % of leaf surface area infected). INA in the future might replace conventional fungides that pollute the environment but there is a necessity to evaluate the costs and other impacts.

Common blight was of minor importance in the Michigan bean production in 1995 (Hart, personal communication). Long periods of drought might have affected the infection of field grown plants after challenge inoculation was. The experiments reported here need to be conducted over several years to determine how the environment influences the INA - bean interaction in the development of SAR.

PART II : PARTIAL CHARACTERIZATION OF BACTERIA ISOLATED FROM AZUKI BEAN PLANTS

I. INTRODUCTION:

During the summer of 1994, bacterial lesions on leaves were observed in commercial fields of Azuki bean grown in Michigan. These lesions were characterized by necrotic areas surrounded by yellow halos and necrosis, and were similar in appearance to halo blight caused by *Pseudomonas syringae* pv. *phaseolicola* and common blight caused by *Xanthomonas campestris* pv. *phaseoli* on Michigan dry bean cultivars. We were concerned that Michigan dry bean cultivars might be susceptible to pathogens causing disease on Azuki bean. Therefore, a study was conducted to identify and characterize the pathogens causing disease on Azuki bean and determine if the host range included Michigan common beans.

To achieve this goal, a series of tests and experiments were conducted in the laboratory and greenhouse. The overall goal of this research was to determine if bacterial diseases of the Azuki bean observed in Michigan fields in 1994 were limited to Azuki bean, or if the Michigan dry bean industry was at risk from potentially new bacterial diseases. Specific objectives of this research were to:

1) isolate and characterize the bacteria associated with the disease, using morphological, biochemical and molecular criteria;

2) complete Koch's postulate and determine the host range of the isolates;

3) compare the isolates with known strains of the pathogens.

II. LITERATURE REVIEW:

Azuki bean (*Vigna angularis)* is an important legume in most countries in the world, especially in the East and Southeast Asia. It belongs to the family of Fabiaceae, tribe Phaseoleae, in the genus of *Vigna*. In the United States, Azuki beans are grown mainly for export, and much of the seed originates in foreign countries. Several species of bacteria including *Curtobacterium, Pseudomonas, and Xanthomonas* have been reported as pathogens of Azuki. In Nebraska, several highly virulent isolates of *Curtobacterium flaccumfasciens* pv *flaccumfasciens* on Azuki bean were reported (Coyne *et al.*, 1963; Bradbury, 1986). Another bacterial disease, bacteria stem rot, caused by *Pseudomonas adzudicola* (Bradbury, 1986) was reported as a host-specific pathogen of Azuki. Bacterial brown stem rot has characteristics closely resembling *Pseudomonas syringae* pv. *glycinea, Pseudomonas syringae* pv *phaseolicola* and *Pseudomonas syringae* pv. *mori* (Tanii and Baba, 1971). Kennedy *et al* 1984 reported some new bacterial diseases of Azuki bean in Minnesota that caused stem rot. Pathogenicity was confirmed in greenhouse inoculations and some cultivars of common bean were susceptible to this pathogen. The bacterial strain resembles *P. adzudicola,* which was described for the first time in 1979 in Japan.

Identification of plant pathogenic bacteria involve different steps including the Gram-stain, the color of the colony on different types of media, the growth habit, the form of the cell, presence of a spore, flagellation, the respiration and differential carbon carbon source for growth. Other important tests include antibody testing, DNA typing, a

pathogenicity test using tobacco to determine the hypersensitive reaction (HR), and host range on other plant species.

Chemical test and morphological characteristics on specific media.

Bacteria are divided in two groups: the Gram - positive bacteria which retain the primary violet dye after washing with alcohol, and the Gram - negative bacteria which lose the violet coloration after alcohol washing and counter staining with safranin. The Gram stain gives a purple to blue-black color for gram-positive bacteria and red color for Gram - negative bacteria (Schaad, 1994). The KOH test (3% KOH) provides a rapid response to determine the gram reaction but it is not always reliable.

Bacteria grown on different media often result in colonies of different color and growth characteristics. For example, on nutrient glucose agar (NGA), yeast extract-dextrose-$CaCO_3$ (YDC), or nutrient-broth yeast extract agar (NBY), the *Xanthomonas* group gives yellow colonies while *Pseudomonas* does not. *Erwinia* grows on Miller-Schroth (MS) medium, but *Xanthomonas* does not. *Pseudomonas* can produce fluorescent pigment on King's B agar, while *Agrobacterium* cannot. *Agrobacterium* is the only group which grows on D-1 agar (Schaad, 1994). The presence of more than four peritrichous flagella or not can help to define the different groups. *Erwinia* has more than four peritrichous flagella. The form or shape of the bacterial cell can also help to determine the bacterial group. The shape can be rod, coccus, bacillus or coryneform. The presence of a spore is a characteristic of the *Bacillus* group and *Streptomyces* have aerial mycelia (Schaad, 1994). Other tests including the use of carbon sources, oxidase reaction

and arginine dehydrogenase are also important for determination of species or pathovars.

Molecular tests: Polymerase Chain Reaction

Chemical and morphological tests are generally sufficient to differentiate species. The use of molecular tests can provide good separation among closely related bacterial groups. Polymerase chain reaction or PCR is one of the most powerful tools because it can provide a DNA fingerprint that can be strain or species specific. PCR is performed by amplifying segments of genomic DNA for different species of bacteria. DNA polymerase uses single-stranded DNA as a template for the synthesis of a complementary new strand (Watson *et al* 1992). The PCR mixture contains buffer, dNTPs, primers, enzyme, *Taq* polymerase and water at desirable concentrations (Watson et al 1992). The DNA template contains the target segment to amplify. A drop of oil is added to the samples to prevent evaporation during the process. The mixture is placed in well of a PCR machine called a thermocycler. The reaction can be completed in 30 to 60 cycles. The starting material is double - stranded DNA. The strands are separated by heating the reaction mixture and then cooled so that the primers anneal to the two primer binding sites that flank the target region. *Taq* polymerase synthesizes new strands of DNA, complementary to the template. *Taq* polymerase improves the sensitivity and specificity of the PCR. The reaction mixture goes through repeated cycles of primer annealing, DNA synthesis and denaturation of DNA fragments. The target sequence doubles in concentration for each cycle.

Pathogenicity tests.

Not all bacterial species are pathogenic to plants. Some are saprophytes even though they are often isolated from infected tissue. The pathogenicity of bacterial isolates can be determined by observing the hypersensitive reaction (HR) on tobacco, or by inoculating different plants of the same family or species (Fulbright personal communication). When plant pathogenic bacteria are injected into the leaf of tobacco, the hypersensitive reaction (HR) occurs (necrosis or death of tissue surrounding the inoculation point). There is no reaction to non pathogenic bacteria.

III. MATERIAL AND METHODS.

Isolation from field infected Azuki bean in 1994

Lesions were observed on Azuki bean leaves and pods during the summer of 1994. Samples of infected leaves and pods were collected by Dr. Patrick Hart for examination. Lesions on leaves were characterized by necrosis and yellowing. The lower surface of leaves exhibited water soaking lesions. Symptoms on pods were also characterized by water soaking lesions. Samples of leaves and pods were surface sterilized with 10% clorox bleach for five minutes. Diseased pieces of leaves were then chopped with a razor blade in a drop of sterile water in a petri dish and small loops of water were serially transferred to additional drops of sterile distilled water. The dilutions were streaked onto nutrient agar plates and placed under a fluorescent light for three to five days. Singles colonies of bacteria (white, yellow and orange) were observed on the medium. These single colonies were restreaked on medium to obtain pure cultures of the bacterial isolates

Isolation from infected seed and seedlings grown from commercial seed obtained in 1995.

A supply of Azuki bean seed (cultivar Erimo) was obtained for pathogenicity tests. Preliminary attempts to grow plants resulted in severe stem rotting and post emergence damping off (Fig. 10 and 11). Therefore, the cause of early death of young seedlings of Azuki bean was also investigated as a possible source of contamination. Seeds were surface with sterilized with 5% clorox for 10 minutes to determine if the contamination

was internal or external. The seeds were crushed in sterile petri plates in sterile distilled water and a drop of the liquid was streaked on nutrient glucose agar. From non- surface sterilized seed, three types of bacteria colonies were obtained (yellow, white and orange) and from surface sterilized seed two types of colonies were obtained (yellow and white).

Treated (10% clorox) and untreated seeds were grown in sterilized clay pots filled with Bacto-Professional planting mix. A total of 10 pots for each treatment was planted with three seeds per pot. Ten days after germination the seedlings were evaluated for disease symptoms and percent of germination.

Fig. 7: Azuki bean seedlings showing symptoms of natural infection by bacteria 10 days after planting (cultivar Erimo).

1= wilting of the seedling, 2 = necrotic lesions on leaves.


1


2

Fig 8: Azuki bean leaves showing necrotic spots and chlorosis from naturally infected seed on cultivar Erimo 10 days after planting.

3 = Necrosis and curling on leaves; 4= Chlorosis on leaves.

3

4

Plants material:

In greenhouse experiments, cultivars of several Michigan edible bean varieties, Azuki beans and cowpea were grown in clays pots filled with Bacto - professional planting mix. A total of six cultivars of common bean (*Phaseolus vulgaris)*, two cultivars of disease-free Azuki bean (*Vigna angularis*), and three varieties of cowpea (*Vigna unguiculata*) all obtained from Dr. J. Kelly's lab in Crop and Soil Sciences Department were used in this study.

Plants were maintained in the greenhouse at the temperature of 20 to 25 ° C during the day and 18 to 21 °C during the night under continuous fluorescent lighting. A total of ten pots per variety with three plants in each pot were grown for the experiments. The characteristics of the different cultivars were the following:

Common bean cultivars:

Cultivar name	**Commercial class**
Mayflower :	Navy
Aztec :	Pinto
Alpine :	GN(Great Northern)
Montcalm :	DRK (Dark Red kidney)
Huron :	
Taylor :	Cranberry

Azuki bean cultivars:

Erimo	UJBU (Japan)
Common	424 (Washington)

Cowpea cultivars:

CB5 :	California's black eye
CB46 :	California's black eye
CB88 :	California's black eye

Bacterial isolates

To evaluate the relative virulence and pathogenicity of bacteria isolated from Azuki bean, known isolates of pathogenic bacteria were used to compare with the isolates obtained from Azuki leaves, seedlings and seed. An isolate of *Xanthomonas campestris* pv *phaseoli* strain D.F. 151 and *Pseudomonas syringae* pv. *phaseolicola*, were obtained from Dr. D. Fulbright. *Curtobacterium flaccumfasciens* sp. *flaccumfasciens*, strain NE 21 was obtained from the University of Nebraska (A.Vidaver). This srain had the same characteristics as the other strains obtained from Nebraska (SWM2 and var. aurantiacun 2A-Lt) In addition to these known pathogenic strains, six isolates of bacteria from Azuki leaves, stem and seed were used in pathogenicity tests on the different cultivars of common bean Azuki bean, and cowpea (Table 10). The medium used to maintain the bacteria was nutrient-broth yeast extract agar: nutrient broth (8 g/l), yeast extract (2.0g/l), K_2HPO_4 (2.g/l), KH_2PO_4(0.5g/l), glucose (2.5/l), agar (15g/l). The morphological charactereistics used for differentiation included the colony morphology, the Gram reaction, the hypersensitve reaction on tobacco and the fluorescent test on King B medium.

Preparation of inoculum and inoculation procedure:

Common bean, Azuki bean and cowpea plants were grown in the greenhouse to the 2-3 trifoliate stage and then incubated in a mist chamber at about 20°C for 24 hours prior to inoculation. Bacterial isolates from single colonies were grown overnight on a nutrient broth medium in a rotary incubator at 27°C and at 150 rpm. Bacterial cells were collected in the log phase. Bacteria were centrifuged at 1 x 10^4 rpm in a 50-ml tube in an IEC-4B centrifuge, and the pellets collected and diluted in sterile distilled water. The $O.D._{600}$ was adjusted between 0.05 and 0.1 using a spectrophotometer.

Two methods of inoculation were used in these experiments. A scion was made at the point the cotyledon was attached to the stem to allow inoculation by introducing bacteria into the cut with a needle and syringe. A second method of inoculation was from the lower side of the cotyledons leaves using a syringe without a needle and placing the leaf between the syringe opening and the thumb to infiltrate the bacterial inoculum into the mesophile of the leaves. Each pot contained 3 plants. The inoculated plants were transferred to a mist chamber, temperature about 20°C for 24 to 48 hours. The plants were then placed on greenhouse benches and watered daily. Each cultivar of common bean, Azuki bean and cowpea was inoculated with the six strains of bacteria isolated from Azuki bean plus the three control pathogens: *X. campestris* pv. *phaseoli*, *P. syringae* pv. *phaseolicola*, and *C. flaccumfasciens* ssp. *flaccumfasciens* (isolate NE 21). Ten days after inoculation, cultivars were evaluated for their reaction to the different strains. The plants were evaluated according to the type of symptoms ranging from a general wilting to leaf necrosis. The experiment was repeated three times and the results are the means for

the three experiments.

Molecular analysis of bacteria strains

All of the bacteria isolates tested for pathogenicity were evaluated by PCR for differences in DNA fingerprints. The PCR box primer was used in this analysis (from Fulbright Laboratory). The preparation of the sample was the first step in the analysis. The primer used in this study was BOX 1AR. The preparation of the master mix (BO X 1AR) (Carmen personal communication) was as follows:

For each sample:

5.0 ul Gitschier Buffer,

0.2 ul BSA,

2.5 ul 100% DMSO

13.5 ul dH_2O

1.0 ul Tween 20 (1%)

1.25 ul 25 mM Mix of dNTP's (1:1:1:1)

1.0 ul primer (0.3 ug/ul = 50 umol/ul)

0.4 ul *Taq* polymerase 2 U Perkin and Elmer.

The total volume of the sample was 24.85 ul. The correct volume of master mixture was distributed to 0.5 ml Eppendorf tubes. For each tube, the master mix was 23.8 ul and 1 ul of sample DNA (50 ng /ul), 25 ul of mineral oil covered the top of the sample. The samples were centrifuged for five seconds and loaded in the thermocycler. The box program was 007-008-009 and there were 35 cycles (see appendix C). After

amplification, 6 ul of the amplified product was mixed with 1 drop (approximatively 1 ul) of tracking dye and the DNA bands separated on agarose gel (Sambrook *et al*). The standard was 1 ul of a 100bp ladder, 1 ul of dye and 5 ul of dH_2O. The gel was run at 81 volts for 18 hours. The gel was stained in Ethidium bromide and a picture taken using / 57-Polaroid film, (light exposure at 8).

IV. RESULTS

Effects of seed surface sterilization on germination and isolation of bacteria:

The surface sterilized seeds of the cultivar Erimo had a higher percentage germination compared to the non-surface sterilized seed. Of 30 surface sterilized seeds planted 29 germinated (96%), but only 24 out of 30 non- sterilized seeds germinated (80%). Seed treatment slightly improved germination. Seedlings were evaluated for disease symptoms 10 days after planting. Early symptoms on seedlings were characterized by stem rotting and leaf puckering (Figs. 10 and 11). Treated and untreated plants exhibited similar symptoms with 75% (22) and 62% (15) of seedlings affected respectively (Table 9). These results suggested that the seed infection was internal as well as external for this paricular lot of seeds.

Table 9: Effect of seed treatment on germination and seedling infection on the Azuki bean cultivar Erimo.

Treatments	treated seeds	untreated seeds
Total seed planted	30	30
germination	29	24
% of germination	96	80
infected seedlings	22	15
% infection	75	62

Results are the means of two experiments. Symptoms appeared earlier on untreated plants and most of the infected seedlings later died.

Characteristics of bacterial strains:

The different strains of bacteria isolated from Azuki bean were compared with known bacterial pathogens of bean (Table 11) in a series of tests. The strains were designated according to their origin and the year of their collection. Two isolates obtained from Azuki bean leaves in Michigan in 1994 from the field were provided by Dr. Hart (KS-94-1 and KS-94-2). KS-94-1 was a yellow Gram - positive strain and KS-94-2 was an orange Gram - positive strain. KS-94-2 did not cause a HR on tobacco. Two isolates from the seed of Azuki bean cultiva Erimo were designated KS-95- and KS-95-2. KS-95-1 was Gram - positive with a yellow colony appearance and KS-95-2 was gram - negative with a white colony appearance. The isolates obtained from the stem of Azuki bean seedlings cultivar Erimo in the greenhouse were designated as KS-95-3 and KS-95-4. These were a white, gram negative, and a yellow gram positive respectively.

The known strain of *X. campestris* pv. *phaseoli* was Gram - negative and produced yellow colonies on YDC or NBY medium (Table 11). This bacteria caused common blight on bean. The strain *C. flaccumfasciens* sp. *flaccumfasciens* was Gram positive bacteria and caused wilting in a number of bean varieties (Tables 11 and 12). It produced yellow to orange colonies on NBY or YDC medium. The strain *P. syringae* pv. *phaseolicola* was gram negative and produced white colonies on different media. It caused halo blight in some edible bean cultivars (table 10).

Table 10: characteristics of bacterial isolates used in inoculation studies of common bean, Azuki bean, and cowpea.

bacteria isolates	origin	colony morphology	Gram reaction	reaction on tobacco	fluorescens test
X.c.phaseoli	Fulbright	yellow	negative	HR	NO
C.f.f. NE 21	Nebraska	yellow	positive	HR	NO
P.s.phaseolic	Fulbright	white	negative	HR	NO
KS- 94-1	Field Mich	yellow	positive	HR	NO
KS-94-2	Field Mich	orange	positive	NO	NO
KS-95-3	Greenhouse	white	negative	HR	YES
KS-95-4	Greenhouse	yellow	positive	HR	NO
KS-95-1	Seed MI	yellow	positive	HR	NO
KS-95-2	Seed MI	white	negative	HR	NO

Note: X.c.p =*Xanthomonas campestris* pv. *phaseoli*; C.f.f = *Curtobacterium flaccumfasciens* species *flaccumfasciens* (Nebraska), KS- 94-1: bacteria isolated from Azuki leaf in 94- isolate #1; KS- 94-2: bacteria isolated from Azuki leaf in 94, isolate # 2; KS-95-3 : bacteria isolate from Azuki stems in 95, isolate #1; KS-95-4 : bacteria isolate from Azuki stems in 95, isolate # 2, KS- 95-1: bacteria isolate from Azuki seed in 95, isolate # 1; KS-.95-2: bacteria isolate from Azuki seed in 95 isolate # 2; *Ps.phaseolic*: *Pseudomonas syringae* pv *phaseolicola.* MI = Michigan

Pathogenicity tests:

All the bacterial isolates tested except one were pathogenic. Symptoms characterized by wilting and necrosis were more frequent than chlorosis. Both stems and leaves were inoculated as described previously. On cowpea, only the strain *C. flaccumfasciens* sp. *flaccumfasciens* from Nebraska was able to cause infection, all other isolates including *X.campestris* pv.*phaseoli* and *Pseudomonas syringae* pv.*syringae* were not pathogenic. KS-94-1, a field isolate from Azuki bean infected all the cultivars of common beans, including Azuki bean. KS-94-2 which was negative on the HR test on tobacco caused minor wilting on Erimo only, and KS-95-2 caused minor necrosis also on Erimo. The other strains had variable reaction on different cultivars (table 11).

Table 11: Comparison of pathogenicity and symptoms on cultivars of common bean Azuki bean, and cowpea inoculated with strains of bacteria isolated from Azuki bean or common bean in greenhouse.

strains	Xcp*	C.ff.N E 21	Psp*	KS-94-1	KS-94-2	KS-95-3	KS-95-4	KS-95-1	KS-95-2
Cowpea									
CB5		wilt	-	-	-	-	-	-	-
CB46	-	wilt	-	-	-	-	-	-	-
CB88	-	-	-	-	-	-	-	wilt	-
Azuki	**bean**								
Erimo	necros.	-	-	necros.	wilt	necros.	wilt	-	necros.
commo	dead	wilt	-	necros.	-	wilt	-	-	-
common	**bean**								
Aztec	wilt,nec	wilt	chloro	necros.	-	wilt	wilt	wilt	-
Huron	necros.	wilt	necros.	chloros	-	-	wilt	-	-
Alpine	wilt,nec	wilt	necros.	necros.	-	wilt	wilt	necros.	-
Montca	necros	wilt	wilt	necros.	-	-	-	-	-
Mayflo	wilt,nec	wilt	wilt	necros.	-	-	wilt	wilt	-
Taylor	wilt,nec	wilt	wilt,ne	necros.	-	-	wilt	-	-

* isolated from edible beans.

wilt = wilting, nec or necros = necrosis, chloros = chlorosis; Mayflo = Mayflower; Montca = Montcalm, (-)= no reaction.

In comparison to known strains, it appeared that none of the bacteria isolates from Azuki were closely related to *Xanthomonas*. The two isolates KS-94-1 and KS-95-1 were similar to *Curtobacterium*. The two isolates KS-95-3 and KS-92-2 were close to the known group *Pseudomonas*, all Gram - negative with white colonies on NBY medium, but only KS-95-3 was fluorescent on king's B medium. The following table provides grouping of the isolates.

Table 12: grouping of different isolates of bacteria

Group 1	Group 2	Group3
Xanthomonas	*Curtobacterium*	*Pseudomonas*
-	KS-95-1	KS-94-3
-	KS-95-1	KS -95-2
-	KS-95-4	

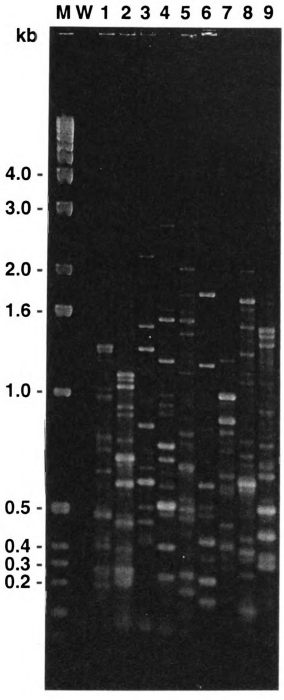
Note.The strain Lea-94-2 did not cause a HR on tobacco and did not belong to any of the known groups.

Molecular analysis.

The DNA fingerprints of the different strains of bacteria used in this study were analyzed to determine if the isolates from Azuki bean were related to the known pathogens of edible bean in Michigan. KS-94-1 and KS-95-1 were very similar in colony color, type of colony and both were gram positive, but showed large differences in bands when their DNA was amplified. However, KS-95-1 shared 2 bands with *C. flaccumfasciens sp.flaccumfasciens*, a gram positve bacteria that caused wilting in edible bean plants. The two isolates with white colonies and gram negative characteristics where similar to *Pseudomonas syringae* pv. *phaseolicola* but their DNA fingerprint was quite different. (Fig 12). The DNA fingerprint showed some similarty in band between KS-94-1, isolated from Azuki leaves and KS-95-4 isolated from Azuki stem .

Fig 9: PCR amplifications of bacteria strain isolated from Azuki bean leaves, seed and stems of seedlings.

PCR results of bacterial strains used in this study showed difference between strains that had the same types of colonies, same color and morphology and different from known strains that caused symptoms on common bean plants M = marker, W = water (control), 1 = Cff, 2 = Cmm; 3 = KS-95-1, 4 = KS-94-1, 5 = Psp, 6 = KS-95-2, 7= KS-95-3, 8= Xcp; 9= KS-95-4.


M W 1 2 3 4 5 6 7 8 9
kb
4.0 -
3.0 -
2.0 -
1.6 -
1.0 -
0.5 -
0.4 -
0.3 -
0.2 -

DISCUSSION AND CONCLUSIONS

The different strains of bacteria found on Azuki bean had morphological characteristics close to some species of bacterial pathogens of common bean in Michigan. The wilting, stem rotting and damping off were the most frequent symptoms on seedlings of Azuki. The bacterial transmission was probably by seed externally and internally contaminated. However, bacteria isolated from seed had different characteristics from those obtained from seedlings or leaves. Symptoms described by Kennedy in 1984 on Azuki bean in Minnesota were similar to those found in this study. Most of the strains identified in this study in Michigan caused necrosis and wilting on other species of bean, but not on cowpea. Two strains KS-94-1 and KS-95-1, were very similar to one strain *C.flaccumfasciens sp. flaccumfasciens* NE 21 from Nebraska. These were all gram positive, yellow colonies on NBY, and caused HR on tobacco. Despite these similarities only the strain KS-95-1 had a DNA fingerprint to close that of *Curtobacterium.* KS-95-4 strain appeared different from the subspecies obtained from Nebraska. KS-94-1 caused necrosis on most of the bean species, while KS-95-1 caused wilting on most species. The pathogenic species described as *Pseudomonas adzudicola* could be closely related to two of the strains as determined by their colony color and their gram stains (KS-95-3 and KS 95-2). These strains were gram negative and had white colonies resembling those of our strains of *Pseudomonas syringae* pv *phaseolicola* (DWF) which are also characteristics of *Pseudomonas adzudicola.* A strain of *Pseudomonas adzudicola* was not availablefor DNA fingerprinting. Therefore, the comparison was not complete. None of the strains

were similar to *Xanthomonas* species. The strain KS-94-2 appears to be a saprophytic bacteria of bean.

The results obtained from the morphlogical, chemical, pathogenicity and molecular tests showed that the strais responsible for the different symptoms on Azuki bean could be the two strains KS-94-1 and KS-95-4. These two strain shared multiple bands in their DNA fingerprint and were pathogenic to most of the cultivars of common bean in greenhouse experiments.

Not all these bacterial strains were pathogenic to the common bean cultivar of *Phaseolus vulgaris* grown in Michigan. The strain KS-94-2 which had orange colonies on NBY did not cause any symptoms on common bean, but most other strains KS-95-1, KS-95-3, KS-95-4 and KS- 94-1 caused symptoms on most bean species when different cultivars were inoculated in the stem or on the leaf surface.

This study reports on the characterization of different trains of bacteria isolated from Azuki bean. Tentative identification of the isolates as *Curtobacterium* and *Pseudomonas* were made but additional tests should be made. Symptoms that developed on common bean cultivars after inoculation with bacterial strains doesn't necessarily mean that common beans grown in Michigan are at risk from pathogenic bacteria of Azuki bean. A state survey to determine if *Curtobacterium* or *Pseudomonas adzudicola* are causing disease on Michigan's dry bean should be considered for future studies.

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APPENDICES

APPENDIX A

Polyacrylamide anionic gel (for native gel preparation): pH 9.3

Lower gel

(1) 30g acrylamide
0.8 g bis acrylamide
Water to 100 ml. Keep in fridge max 1month.
(2)18.5 g tris base
2.1 ml concentrated HCl or 24 ml 1M HCl
0.24 ml TEMED
water to 100 ml
(3) 10% ammonium persulfate. Make fresh weekly, keep cooled
for 1.5 mm gel mix 20 ml water, 0.28 ml ammonium persulfate (3) 10 ml acrylamide mix, 10 ml Tris mix (2).
Pour overlay water. Set for 40 mn. Pour off water.

Stacking gel.

(5) 10 g acrylamide
0.8 g bis acrylamide
Dilute to 100 ml of water, keep in fridge for one month.
(6) 2.23 g tris base
12.8 ml 1M H_3PO_4
0.1 ml TEMED.
For 1.5 mm gel, mix 10 ml of water, 70 ml ammonium persulfate (3), 5 ml acrylamide mix (5) 5 ml Tris mix (6).
Pour set in a comb approximately for 3 hours.

Upper buffer.

5.16 g Tris base
3.48 g glycine
water to 1000 ml pH = 8.9

Lower Buffer.

14.5g Tris base
5.2 g concentrated HCl or 60 ml 1N HCl
Water to 1000 ml.
Load. Run to 50 V.

For staining:
4-dichloro-1-naphtol (120 mg)
140 ml ddH_2O
40 ml methanol
20 ml 10 X PBS pH 7.3
40 ul H_2O_2 (hydrogen peroxide), addition 0.36 ml of H2O2.
In few minutes bands start to appear.

Coomassie blue- stain preparation.

For 1 liter:
0.25 g Coomassie R-250 (brilliant blue)
400ml methanol
stir until dissolved
Add 70 ml acetic acid (glacial)
Add 530 ml H_2O
Filter through whatman # 1 filter paper using a funnel under gravity.
For overnight staining: 4 parts of stain (80%): 1 part of destain (20% solution I)

Destain solution I:

500 ml methanol.
100 ml acetic acid
400 ml H_2O

Destain solution II:

70 ml acetic acid glacial
50 ml methanol
880 ml H_2O.

APPENDIX B

Protocol\SDS-PAGE.
RECIPES FOR SDS-PAGE.
References: B.D Hames and D. Rickwood , editors, 1981, " Gel electrophoresis of proteins : a practical approach", IRL Press.

Running gel: (10 ml)	6.5%	7.%	7.5%	8%	9%	10%	12%	13%	15%	
Acrylamide	2.17	2.3	2.5	2.7	3.0	3.3	4.0	4.3	5.0	ml
1.75 M Tris pH 8.8	2	2	2	2	2	2	2	2	2	ml
10%SDS	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	ml
Water	5.66	5.5	5.3	5.13	4.8	4.53	3.83	3.5	2.83	ml
TEMED10%	10	10	10	10	10	10	10	10	10	ul
ammonium persulfate	60	60	60	60	60	60	60	60	60	ul

Stacking gel:
(6 ml)

1. Acrylamide	1.0 ml
25 M Tris pH 6.8	0.6 ml
10% SDS	60 ul
Water	4.3 ml
TEMED10%	5 ul
Ammonium persulfate	50 ul

Prior to adding TEMED and ammonium persulfate, degas the combination for 15 minutes,
Acrylamide (30:08) = 30 gm acrylamide, 0.8 gm bis-acrylamide per 100 ml.
Filter through whatman # 1.
1.75 M Tris = 21.2 gm / 100 ml pH 8.8
1.25 M Tris = 15.1 gm / 100 ml. pH.6.8

Running Buffer: (per liter)

Tris base	3.0 gm
glycine	14.4 gm
SDS	1.0 gm

Sample Buffer: (2 x) (4 X)
(To make 25 ml)

1.25 M Tris -Hcl, pH 6.8	2.5 ml
mercaptoethanol	2.5 ml
[or DDT	20 Mm]
SDS	10 ml 10%
Sucrose	5.0 gm
Bromophenol blue	1.0 gm (add until color looks right
Water	to 25 ml (10 ml) (6 ml).

APPENDIX C

PCR protocol for box primer:
Primer: (BO X IAR)
Preparation of Master Mix:
5 ul Gitschier Buffer
0.2 ul BSA
2,5 ul 100% DMSO
13.5 ul H_2O
1.0 ul Tween 20 (1%)
1.25 ul 25 mM Mix of dNTP's (1:1:1:1)
1.0 ul primer (0.3 ug/ul -- 50 umol/l)
0.4 ul Taq polymerase 2 U Perkin Elmer.

Prepare per tube:

Master mix	24 ul
DNA (50 ng/ul)	1 ul
Mineral oil	25 ul

Spin for 5 seconds and load in PCR machine.

The program applies to thermocycler in room 34 (D. Fulbright).

	program #	for BOX	
1 X	007	95o C	7 minutes
35 X	008	94o C	1 minutes
		53o C	1 minutes
		65o C	8 minutes
1 X	009	65o C	15 minutes
		4oC	Soak

Load 6 ul of PCR preparation per sample onto gel.
Samples are mixed with 2 ul of blue dye before loading.
1 ul of Marker (ladder) is used for the size of the bands

Preparation of gel for electrophoresis:
Agarose 3.75 gm
0.5 X TAE Buffer 250 ml
The mixture is placed in a small autolave with vapor pressure for 15 minutes and cooled down using a stirrer (a magnetic bar) until ready to pour in a comb tray until it solidifies.

Appendix D:

Morphological and chemichal tests of bacteria isolated from Azuki bean leaf in 1994.

Strains/test	Xcp	Pss	P. Fluores	KS-94-1	KS-94-2
Gram test	-	-	-	+	+
Motility	+	+	+	+	+
aeration	+	+	+	+	+
pigment	yellow	white	white brown	yellow	orange
shape	rod	rod	rod	coryneform	rod
growth 37oC	fast	moderate	very fast	very fast	slow
oxidase test	-	-	+	-	-
ADH tets		-	+	+	-
PAF	+ (brown)	+	+	+	+
YDC	+ (brown)	+	+	+	+
no carbon	-	-	-	-	-
sucrose	+	+	+	+	+
Mannitol	+	+	+	+	+
D. Tatrate	-	+	-	-	+
L. Lactate	+	+	-	-	-
Sorbitol	-	+	+	+	-
Tobacco HR	+	+	+	+	+
4 '0 clock	-	-	-	-	-
Bunsi	+	+	-	+	-
Midland	+	+	-	+	-
Mayflower	+	+	-	+	-
Azuki bean	+	+	+	+	+
Fluorescent	-	+	+	-	-