RHIZOSPHERE COMPETENCE, ANTIBIOTIC AND SIDEROPHORE BIOSYNTHESIS IN *PSEUDOMONAS CHLORORAPHIS*: IMPLICATIONS FOR THE BIOLOGICAL CONTROL OF COTTON SEEDLING DISEASE PATHOGENS

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RHIZOSPHERE COMPETENCE, ANTIBIOTIC AND SIDEROPHORE BIOSYNTHESIS IN *PSEUDOMONAS CHLORORAPHIS*: IMPLICATIONS FOR THE BIOLOGICAL CONTROL OF COTTON SEEDLING DISEASE PATHOGENS

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(ABSTRACT)

Cotton seedling disease caused by *Pythium ultimum* Trow and *Rhizoctonia solani* Kühn occurs worldwide in soils ranging from pH 4.5 to 8.5. Studies with cotton have not yet established the relative importance of two classes of secondary metabolites synthesized by soilborne pseudomonads, siderophores (sid) (low molecular weight Fe^{+3} chelators) and antibiotics (ant), in the suppression of these pathogens. Greenhouse bioassays to screen for rhizosphere competent strains identified a single strain of *Pseudomonas chlororaphis* (L-850), that produced siderophores and multiple antifungal antibiotics, including one or more phenazines. A Tiff Image Analyzer (TIA) software program was developed that allowed assessment of wild-type (wt) L-850, and (*ant*) and (*sid*) mutant populations as a function of cotton root surface area (cm^2) in the absence of soil irrigation. Bacterial density and distribution patterns on roots evaluated 22, 36, and 50 DAP, in two pathogen-free soils (pH 5.7, high Fe^{+3}, high phosphorus (P); pH 8.0, low Fe^{+3}, low P) indicated that populations of both wt and mutants persisted after day 22 at levels between log 4.6 (lower laterals) to log 6 cfu/cm^2 (upper tap) even as total root area increased 122% from day 22 to 50. Population densities of all strains were consistently 1/8 to 3/4 log unit lower in the pH 5.7 soil on the lower tap and upper lateral roots, respectively. The loss of siderophore production appeared to enhance the rhizosphere competence of strain L-850. For greenhouse trials with three pathogen inoculum densities (low, intermediate, high) protection against pre- and postemergence damping-off (phase 1) and hypocotyl/root rot of young plants (phase 2) by the (*sid*) and wt strains was similar (P = 0.05) whereas, protection

by seed treatment with the (*ant*) mutant was reduced. The level of suppression provided by L-850 was equivalent ($P = 0.05$) to the standard fungicide at low and intermediate pathogen pressure. These studies demonstrated a minimum contribution of siderophores in the biological control of cotton seedling disease and established a significant role for antibiotic biosynthesis over a range of soil physical and chemical characteristics.

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INTRODUCTION

Pythium ultimum Trow. and *Rhizoctonia solani* Kuhn. are important damping-off pathogens causing annual losses on cotton (*Gossypium hirsutum*) in excess of 26 million dollars. Over a thirty-five year period, annual yield losses were estimated to range from 1.0 to 6.5%, even with application of currently registered fungicides. Control of these pathogens has become increasingly important in Virginia in view of the increase in cotton acreage from less than 500 acres in 1987 to 103,000 acres in 1995. Fluorescent soilborne *Pseudomonas* species offer a potential alternative to current chemical control of cotton soilborne diseases due to their suppression of a wide range of fungal pathogens and lack of environmental toxicity. Their development as microbial pest control formulations would reduce the reliance on chemical control, result in lower input costs, and minimize environmental contamination.

The production of two major classes of secondary metabolites, siderophores and antibiotics, by pseudomonads has been proposed as the primary mechanism responsible for the suppressive activity against *P. ultimum* and *R. solani*. Siderophores are low molecular weight fluorescent compounds, produced under iron limiting conditions that chelate iron in the root environment, rendering it unavailable for fungal pathogen growth. Antibiotics, *e.g.*, phenazines and phloroglucinols, function as fungistatic or fungicidal substances. In addition to the production of siderophores or antibiotics, an equally important prerequisite for

successful biological control strains, and one that is frequently relegated to a position of secondary importance in screening procedures, is rhizosphere competence (root colonizing ability). Rhizosphere competence is a critical component of suppressive activity in that synthesis of secondary metabolites must be of sufficient concentration (bacterial cell density dependent) and production must occur at the appropriate pathogen infection sites (cell distribution dependent).

The objectives of this research were: i) to screen twenty *Pseudomonas* biocontrol isolates (Allied and Ecogen culture collection) for a superior cotton rhizosphere competent strain, ii) to assess rhizosphere competence of the isolate selected above at two soil pH extremes (pH 5.7, 8.0) in order for suppression to be operable across a wide range of soil pH, iii) to genetically modify and characterize antibiotic-negative (*ant*) mutants of the isolate selected in part (ii), iv) to compare wild-type, siderophore-negative (*sid*) and antibiotic-negative (*ant*) strains for differences in cotton root population density and distribution patterns, and v) to assess the contribution of antibiotics and siderophores to pathogen suppression under both acid (high iron, high phosphorus) and alkaline (low iron, low phosphorus) soil conditions. Such studies will provide insight into the relative importance of colonization, siderophores, and antibiotics in the biocontrol of cotton seedling pathogens by fluorescent pseudomonads. The results obtained herein may lead to the genetic improvement or enhancement of these microorganisms as biofungicidal seed treatments.

II.

LITERATURE REVIEW

MECHANISMS INVOLVED IN BIOLOGICAL CONTROL BY MEMBERS OF THE GENUS *PSEUDOMONAS*

Fluorescent pseudomonads found in soil and plant rhizospheres comprise the majority of bacteria that are responsible for plant growth promotion and biological disease control in agronomic systems. Important bacterial traits with a proposed involvement in biocontrol and plant growth promotion include adherence (16), motility (17), tolerance to adverse environmental conditions (56), production of secondary metabolites (antibiotics, siderophores; 22,47,52,53,62,83), efficient root colonization (15,56,66,93,94), utilization of specific root exudates minimally used by other indigenous rhizosphere microflora (73,84), and/or enhanced growth rates on exudates metabolized by a broad range of organisms (10).

Rhizosphere Colonization

Colonization of the host plant rhizosphere is an important prerequisite for successful suppression of root disease pathogens by bacterial biocontrol strains. Attributes relevant in selecting for competent root colonizers have included production of secondary metabolites that inhibit indigenous microbes competing for nutritionally favorable sites on the root, and tolerance of variable soil pH, temperature and matric potential (66). Utilization of both water soluble root and volatile seed exudates has been shown to enhance establishment of rhizosphere populations of introduced strains (63,67,68). Soluble exudates include sugars,

amino acids, organic acids, flavonoids, sterols, vitamins, and proteins (63). Seed volatiles scavenged by microbes include ethanol and acetaldehyde (66).

Competition between *Pseudomonas* and *Pythium* species for pea (*Pisum sativum*) and soybean (*Glycine max*) seed volatiles was studied as a factor involved in reduction of damping-off (67,68). Treatment of seeds with *Pseudomonas putida* N1R reduced concentrations of ethanol and acetaldehyde which were produced faster and diffused farther from the seed source than water soluble exudate materials. Elad and Chet (18) provided evidence for nutrient competition in the rhizosphere as a mode of action involved in the control of damping-off and the inhibition of *Pythium aphanidermatum* oospore germination by six *Pseudomonas* isolates. However, the production of antifungal metabolites operating as a secondary mechanism in the biocontrol of *P. aphanidermatum* was not thoroughly investigated.

A research area currently receiving attention is the exploitation of bacterial strains genetically engineered to utilize a single carbon source unique to the rhizosphere environment (46) or growth on multiple carbon/energy substrates (73) already present or exogenously supplied to the root/soil interface with the biocontrol agent. An efficient root colonizing strain of *Pseudomonas fluorescens* isolated from sugarcane (*Saccharum officinarum*) was engineered to utilize sucrose as the sole carbon source, and resulted in an increased ability to colonize sugarcane roots (84). Cloned genes whose products degrade plant components such as cellulose, pectin, xylan and proteins are currently under investigation for

insertion into efficient root colonizing pseudomonads (84). A *lacZY* insertion into a *Pseudomonas aureofaciens* isolate resulted in a lactose-utilizing derivative that was evaluated in studies of population dynamics, long-term persistence and relative competitiveness on fieldgrown wheat (*Triticum aestivum*) roots (46). Bacterial populations of a furrow-applied suspension reached a maximum at 2 wk after planting with lateral and vertical soil dissemination limited to 18 and 30 cm, respectively, after 31 wk. After four successive growth cycles on radish (*Raphanus sativus*), *Pseudomonas putida* strain WCS358, an isolate capable of exploiting a large number of heterologous siderophores produced by other pseudomonads, was able to decrease populations of 4 of 6 indigenous *Pseudomonas* species in rhizosphere competition studies (73). The utilization of additional rhizosphere carbon substrates over that of strain WCS358 was offered as a possible explanation for the lack of decrease with the remaining two bacterial strains.

The effect of soil moisture on the pattern of distribution of rhizosphere-inhabiting pseudomonads has been the subject of numerous studies (4,18,54,66). Bowen and Rovira (10) proposed thee dispersal methods of bacterial populations in the rhizosphere: i) motility in the film of water on the root surface; ii) convection in films of water along the root surface; or iii) passive transport on both the root apex and elongating cells or on fungal hyphae. Howie et al. (35) questioned the role of bacterial motility, suggesting that root colonization occurred by passive transport on the root apex followed by multiplication along the root surface once

cell deposition occurred. This passive transport was observed with a fluorescent *Pseudomonas* strain that colonized wheat roots.

More recent investigations with *Pseudomonas* species on different crop plants have focused on the importance of soil water percolation as an effective means of establishing bacterial populations in the rhizosphere or along root surfaces. Studies by Parke et al. (66), monitoring the movement of *P. fluorescens* 2-79 along wheat roots with and without surface irrigation, indicated that vertical flow following irrigation was more important for bacterial dispersal over long distances on the roots than transport by elongating root tips. Root platings demonstrated the absence of bacteria at the root apex after 4 days in moist soil without additional irrigation. Spatial-temporal colonization patterns of pseudomonads on potato (*Solanum tuberosum*) (4) and matric potential studies with pea (54) further support the importance of vertical soil-water movement in the distribution of bacteria on plant roots.

Potato root tips growing from bacterized seed pieces were sporadically colonized with low populations in the absence of added soil water, whereas irrigation established a declining population density gradient along the entire root system (4). Likewise, a *P. fluorescens* isolate applied to pea seeds was detected on only 5% of root segments 4-5 cm below the seed after 7 days at a steady-state soil matric potential of -1 kPa (54). Irrigation 4 days after planting, extended the detection limits of the bacterium to 9-10 cm below the seed at a population level of 3.06 log cfu. Davis and Whitbread (15) studied plant-growth-promoting

rhizobacteria (PGPR) on radish roots and found that adequate soil moisture alone was not sufficient to establish high bacterial populations and hence growth promotion. Rather, soil percolation was a more critical factor.

Less characterized factors involved in root attachment and enhanced colonization by fluorescent pseudomonads and other soil-inhabiting bacteria include cell surface molecules and morphological structures such as ferric siderophore receptor proteins (73), adhesion proteins (16), flagella (17), pili (fimbriae) (88), and plant root agglutinin recognition sites on the bacterial cell surface (3). Transfer of the pseudobactin 358 receptor protein A gene (*pupA*) from *Pseudomonas putida* WCS358 to *P. fluorescens* WCS374 conferred on this strain the ability to metabolize ferric pseudobactin 358. Subsequent interaction studies under iron-limiting conditions with transformed *P. fluorescens* WCS374 and indigenous radish rhizosphere-inhabiting microbes indicated that harboring of the *pupA* receptor protein conferred a competitive advantage evident by the stable population densities of WCS374 after four successive growth cycles of radish (73). The role of adhesion in survival and colonization was examined by DeFlaun et al. (16) though the use of adhesion deficient mutants of *P. fluorescens* PfO-1, that were also deficient in flagella. Their study demonstrated that attachment to soil and plant surfaces was due to a 34 kDa adhesion protein on the flagellum and not the flagellum itself. Evidence for lack of flagella attachment was obtained with an adhesion-deficient mutant with increased numbers of flagella over that of the wild-type. In contrast, De Weger et al. (17) using immunological techniques,

supported a role for motility in colonization because non-motile mutants of a *Pseudomonas* PGPR strain were impaired in their ability to colonize potato roots. Anderson et al. (3) addressed the role of agglutination in root colonization, when they compared colonization of Agg(-) mutants with parental Agg(+) *P. putida* cells on kidney bean (*Phaseolus vulgaris*) roots. Agg(-) cells adhered to bean roots at levels 20- to 30-fold less than the parental Agg(+) strain. Although pili-mediated attachment of bacteria to plants has been demonstrated for numerous bacterial genera, Vesper (88) first reported their occurrence on *P. fluorescens* strains. The presence of pili correlated with the ability of the bacterium to attach to corn (*Zea mays*) roots.

Root colonization alone is insufficient for successful biological control of root pathogens unless it is accompanied by an ability to occupy and maintain sufficient population levels along the root at likely pathogen infection sites. The dynamics involved depend on the particular host-pathogen system. A critical consideration is the duration of protection period, (*e.g.*, seedling stage pathogens such as *Pythium* and *Rhizoctonia* where the length of pathogen suppression required is relatively short-term) or more long term protection of plants against *Fusarium* spp. or *Verticillium spp.* (pathogens that can attack plant roots throughout the growing season). Studies to date have shown a high degree of variability in spatial-temporal population data for the same biocontrol strain on different host plants, due in part to dissimilar soil environmental factors (reviewed in ref. 94) or the use of sterile (gnotobiotic) versus natural soil test conditions (8). Bacteria applied to

seeds in autoclaved soil reached a population level on the roots that varied with the bacterium-plant combination tested, regardless of the initial bacterial dose (8). In duplicate studies under nonsterile conditions, competition from indigenous root microflora reduced population levels of the test isolates. Osburn et al. (65) investigated population levels and dynamics between *P. ultimum* and several *Pseudomonas* species on sugar beet (*Beta vulgaris*) seeds under natural soil conditions. Under favorable conditions, protection against *P. ultimum* colonization of sugar beet pericarps was critical during the first 4 h after planting. An antagonistic interaction occurred between *P. ultimum* and the bacterial strains during that period, indicating sufficient population density and distribution at the appropriate infection sites. Threshold population levels for disease control were established for *R. solani*-induced damping-off of cotton seedlings (85), where populations of *P. fluorescens* (4.3×10^6 cells/seed) were required for effective suppression of *R. solani* during the first thee days, the period of highest susceptibility to infection.

Antibiotics

Pseudomonas species produce a wide range of antibiotics that have been implicated in the suppression of numerous soilborne pathogens. In the past two decades researchers have focused on those antibiotics of known structure and broad-spectrum activity, including phenazines (11-13,39,61,70,81-83), 2,4-

diacetylphloroglucinol (20,30,41,42,77,78), pyrrolnitrin (19,31,33,74,75), and pyoluteorin (34).

Phenazine compounds are pigmented, nitrogen-containing heterocyclic antibiotics produced by pseudomonads via the shikimic acid pathway, the branchpoint intermediate being chorismic acid (83,86). Genetic studies with mutants unable to produce phenazines (*phz*) have implicated anthranilate as a phenazine precursor in *P. fluorescens* 2-79 (83). Mutations in the *phzP* biosynthetic gene prevented accumulation of anthranilate when grown under iron-limiting conditions, in contrast to the wild-type strain where anthranilate accumulated. Possible modes of action by phenazines on fungi include inhibition of RNA synthesis, production of toxic superoxide and peroxide molecules (86), or the disruption of energy-requiring, membrane-associated metabolic processes (5). No physiological function for phenazine production by bacteria has been clearly demonstrated although contributions to ecological competence (61) and survival in natural environments (70) have been proposed.

Direct evidence for the production of phenazine-1-carboxylic acid (PCA) in the rhizosphere of wheat following seed application of *P. fluorescens* strain 2-79 was demonstrated by Thomashow et al. (82). PCA was isolated from wheat roots (with adhering soil) in both greenhouse and growth chamber studies at concentrations ranging from 27 to 43 ng/g root tissue. Transposon-generated mutants of *P. fluorescens* 2-79 deficient in PCA production (12,61,81) were no longer suppressive to *Gaeumannomyces graminis* var *tritici* (*G.g.t*), the take-all

pathogen. Colonization of wheat roots and biological control of *G.g.t* with strain 2-79 varied between wheat fields (94), the variability attributed to the existence of soil mineral-level optima for PCA accumulation (79), or loss of biological activity of PCA by ionization in alkaline environments (> pH 7) (11). The genetics involved in biosynthesis of phenazine antibiotics have been characterized for *P. aureofaciens* strain 30-84 (69,70). Regulation of phenazine biosynthesis was found to be similar to the cell density-dependent transcriptional two-component activator system controlling the *lux* operon in *Vibrio fischerii* (70). Knowledge of transcriptional control will assist in determining soil environmental factors that may influence gene expression.

Phloroglucinol antibiotics (phenolic secondary metabolites synthesized by several strains of fluorescent pseudomonads most likely via the polyketide pathway, 78) have been shown to have antiviral, antibacterial, antifungal, antihelminthic, and phytotoxic properties (reviewed in ref. 22). The role of 2,4-diacetylphloroglucinol (Phl) in the control of black root rot of tobacco (caused by *Thielaviopsis basicola*) by *P. fluorescens* strain CHA0, has been extensively characterized (20,41,42,60). In these studies mutants of strain CHA0 that were defective in Phl production afforded a significantly lower degree of plant protection than the wild-type strain. As with the antibiotic PCA, Phl was produced in the rhizosphere at concentrations ranging from 0.94 to 1.36 µg/g root tissue in a gnotobiotic tobacco (*Nicotiana glutinosa*) system (42). Tests with the purified antibiotic *in vitro* however, showed that Phl at a concentration of 160 µM had a

minimal effect on percentage germination of *T. basicola* endoconidia, but completely inhibited germination of tobacco seeds.

Work by Shanahan et al. (77) and Fenton et al. (20) with *Pseudomonas* spp. strain F113 demonstrated the inhibitory activity of Phl against *P. ultimum*, a sugar beet pathogen. Transfer of genes for Phl biosynthesis to *Pseudomonas* spp. strain M114 resulted in increased emergence of bacterized sugar beet seeds compared with wild-type M114 (20). Of seven *Pseudomonas* strains inhibitory to the take-all fungus in growth chamber and field studies, four strains were also positive for Phl production, indicating a role for this antibiotic in the suppression of disease symptoms (30,89). Evidence to date for the mode of action of Phl has included increased production of toxic, oxygenated metabolites such as peroxides and superoxides, as evidenced by increased synthesis of the enzymes catalase and superoxide dismutase respectively, and melanin-like compounds in fungi (reviewed in ref. 53).

Three loci involved in the production of Phl have been identified in studies with *P. fluorescens* strain CHA0 (48). The *gacA* (global antibiotic and cyanide) locus functioned not only as a regulator of Phl, but pleiotropically blocked all secondary metabolites with antifungal activity, thus acting as a global regulator.

Although the structure and function of PCA and Phl have been extensively studied at the molecular level, mutational analyses have implicated other metabolites such as pyrrolnitrin, pyoluteorin, and oomycin A as contributors to the biocontrol activity observed with several important damping-off pathogens

(31,32,37,47). The severity of radish damping-off caused by *R. solani* was reduced 50% by treating seed with either *P. cepacia* or purified pyrrolnitrin (31,32). Two additional pseudane compounds isolated from *P. cepacia*, HMQ [2(2-heptenyl)-3-methyl-4-quinolinol], and NMQ [2(2-nonenyl)-3-methyl-4-quinolinol], exhibited little or no effect on plant protection. Roitman et al. (74,75) identified pyrrolnitrin and four other phenylpyrrole derivatives with antifungal activity against several apple foliar pathogens from a *P. cepacia* bacterial strain isolated from the apple leaf phylloplane. Sequential production of all five compounds was shown to be altered by culture media, pH, and incubation time.

Direct evidence linking pyrrolnitrin and the antifungal activity induced by *P. fluorescens* strain Pf-5 against *Rhizoctonia solani* was obtained by Howell and Stipanovic (33) with cotton seed-soil studies. Seed treatment with either Pf-5 or purified pyrrolnitrin increased seedling survival from 30 to 79%, respectively. Genetic analysis with *P. fluorescens* BL915 which synthesizes multiple antifungal factors (pyrrolnitrin, chitinase, cyanide) with activity against *R. solani*, demonstrated that expression of these compounds was governed by a global regulatory circuit similar to that operating in *P. fluorescens* CHA0 (23).

In an attempt to improve protection by *P. fluorescens* CHA0 against *P. ultimum* damping-off of cucumber, Mauerhofer et al. (60) constructed a pyoluteorin (Plt) overproducing derivative. The Plt derivative showed improved protection of cucumber (*Cucumis sativus*) against *P. ultimum* but was phytotoxic to growth of cress (*Lepidium sativum*) and corn. Contrary to the findings of Maurhofer, Howell

and Stipanovic (34) did not observe phytotoxic symptoms on cotton treated at planting with *P. fluorescens* Pf-5 or purified Plt. Seedling survival increased from 28 to 71% with Plt or Pf-5, respectively.

The antibiotic oomycin A is a unique compound wherein the genes for biosynthesis, and the regulation of those genes have been elucidated, but the structure remains unknown. (37,38). *P. fluorescens* HV37aR2, a rifampicin-resistant derivative of the wild-type strain, and isogenic antifungal (*afu*) mutants were examined for a role in suppression *in vivo* of *P. ultimum* infection on cotton (37). Expression of the *afuE* operon is required for oomycin A biosynthesis. Construction of an *afuE-lacZ* or an *afuE-lux* reporter system in strain HV37aR2 resulted in an indirect correlation of antibiotic production *in vitro* with biosynthesis and biological control *in vivo* (36). Gutterson and colleagues (27) also constructed an oomycin A overproducing strain of HV37a by fusion of an *E. coli* tac promoter to the *afuE* operon and obtained improved suppression of *P. ultimum* damping-off symptoms on cotton.

Siderophores

Siderophores are a group of secondary metabolites of low molecular weight, produced by fluorescent pseudomonads under iron-limiting conditions, that chelate ferric ions (Fe^{+3}) with high specific activity for transport into the cell (9,52). Within the various fluorescent *Pseudomonas* species, genes for siderophore biosynthesis are dispersed throughout the genome with several large regions of clustered genes

(59). The advent of bacterial mutational techniques have allowed researchers to create and contrast siderophore-producing and non-producing strains. Reporter constructs are available that function as biological sensors of Fe^{+3} availability in microbial habitats (58). These techniques have provided evidence in favor of siderophore-mediated iron deprivation in pathogen suppression (6,7,50), or have demonstrated a limited benefit for their synthesis (29,47,57,68). Studies over the past 15 years have shown that competition for iron played a role in the biological control of select soil pathogens but only when chemical and physical factors in the soil were favorable for siderophore production and activity (22). Since iron availability and uptake appear to be critical for production of many antifungal metabolites, the role of competition for iron and its effects on the biosynthesis of a single metabolite becomes difficult to characterize.

Early evidence in support of pyoverdines (Pvd) or pseudobactins, siderophores produced by fluorescent pseudomonads in pathogen-suppressive soil systems, came from studies using traditional non-molecular techniques (44,62,76). Competition for iron between *P. putida* and the flax (*Linum usitatissimum*) wilt pathogen *Fusarium oxysporum* f. sp. *lini* was suggested as the mechanism responsible for Fusarium-suppressive soils in California (76). Addition of *P. putida* isolated from suppressive soil, the iron chelator EDDHA (ethylenediaminedi-O-hydroxyphenylacetic acid) or its ferrated form (FeEDDHA), to pathogen-conducive soil induced a reversion to the suppressive state (76). Suppressiveness was not induced by the chelators DTPA and FeEDTA, both of which have lower iron

stability constants than FeEDDHA or the siderophore synthesized by the pathogen. More recent studies with Fusarium-suppressive soils in France and California attributed suppressiveness to a combination of pseudobactin 358 produced by *P. putida* WCS358 and a non-pathogenic isolate of *F. oxysporum* (50,51). A sequential two-mechanism model was proposed as a result of *in vitro* bioassays, wherein the concentration of available Fe^{+3} was decreased by pseudobactin 358, rendering the pathogenic *F. oxysporum* isolate more sensitive to glucose competition from the non-pathogenic *F. oxysporum* isolate. Becker and Cook (7) implicated siderophores in the suppression of *P. ultimum*, a root rot pathogen of wheat, and in growth-promotion of wheat plants. Siderophore-deficient mutants (*sid*) mutants failed to inhibit *P. ultimum in vitro*. Moreover, no significant growth response occurred on wheat in natural soils following treatment with *(sid)* mutants. In contrast to the majority of studies implicating siderophores as iron scavengers that deplete available iron in the rhizosphere, Ahl et al. (1) proposed siderophores as mediators of iron toxicity. Free Fe^{+3} was found to be less toxic than siderophore-bound Fe^{+3} to *T. basicola*, the black root rot pathogen of tobacco and cotton, on gnotobiotically grown plants.

Contrary to evidence presented by Weller (94), which credited pyoverdines with a role in suppression of the take-all pathogen, *G.g.t*, Hamdan et al. (29) generated mutants of *P. fluorescens* 2-79 deficient in siderophore production that were as effective in controlling *G.g.t.* as the parental strain. However, mutants deficient in PCA were less suppressive than the wild-type strain 2-79. An

additional antifungal factor (Aff), anthranilic acid, played a minor role in suppression, further emphasizing the complexity in characterizing the role of individual secondary metabolites under iron-limiting conditions. Siderophore production by *P. putida* strain N1R was not critical in the control of *P. ultimum* damping-off of cucumber in studies comparing *(sid)* mutants and the parental strain in three different agricultural soils (68). *P. ultimum* sporangial germ tube elongation was sensitive to exogenous soil iron levels 12 h following sporangial germination. However, sporangia had germinated and colonized the seed coat of cucumber within a few h after planting, rendering siderophore-mediated iron deprivation an unlikely biocontrol mechanism. A *pvd* mutant of *P. fluorescens* CHA0 was described that protected tobacco against *T. basicola* to the same extent as did the wild-type strain (40). Results obtained with the mutants indicated that suppression of *T. basicola* in fact required iron.

Although the majority of evidence presented thus far supports a role for secondary metabolites in the biocontrol efficacy of fluorescent pseudomonads, Kraus and Loper failed to identify a role for secondary metabolite production in the biocontrol of *P. ultimum* and *R. solani* (47). They used single-insertion transposon mutagenesis to obtain *P. fluorescens* Pf-5 mutants deficient in various antifungal metabolites. Pyoluteorin (*plt*), pyoverdine (*pvd*), and *apd* mutants (the latter deficient in pyoluteorin, pyrrolnitrin, hydrogen cyanide, and an unidentified compound -- antibiotic 3), all behaved similarly to the wild-type strain Pf-5 in suppressing *P. ultimum* damping-off of cucumber *in situ* and *R. solani in vitro*.

Volatile Compounds

The importance of volatile compounds in the biocontrol of plant pathogens by bacteria has centered on production of the secondary metabolite hydrogen cyanide (HCN) (40,90), or bacterial consumption of seed volatiles that stimulate germination of dormant soil fungal propagules (63,68). Ahl et al. (1) demonstrated that growth inhibition of *T. basicola* was due to cyanic acid synthesis by *P. fluorescens* CHA0, a hypothesis further supported with evidence by Voisard et al. (90), who used an HCN mutant (*hcn*) constructed by a gene replacement technique. Genes involved in HCN production were inserted into a non-inhibitory *P. fluorescens* strain P3 which subsequently demonstrated improved ability to protect tobacco from *T. basicola* but not to the extent of the wild-type strain CHA0. Mechanistic studies implicating the induction of host plant defense genes in response to cyanide stress have been refuted in favor of direct antagonism. *A gacA* mutant of *P. fluorescens* CHA0 deficient in cyanide production was able to induce pathogenesis-related proteins and systemic resistance to tobacco necrosis virus as effectively as the wild-type strain (reviewed in ref. 22). Cyanogenesis is stimulated by Fe^{+3} and as such, appears to be a viable biocontrol mechanism only in iron-rich soils (41).

Induced Resistance

Soilborne *Pseudomonas* species which exert a beneficial effect on crop development have been coined plant growth-promoting rhizobacteria or (PGPR)

(43). Research with PGPR strains has provided some insight into the mechanisms of action by such strains, namely growth promotion and biological control in non-sterile soil. No single mechanism has been positively correlated with plant growth promotion, and the current conclusion is that biological control by PGPR involves the production of secondary metabolites that reduce populations of both root pathogens and deleterious rhizosphere microflora (45).

Recently, direct evidence has mounted implicating PGPR strains of *Pseudomonas* in induced resistance or induced systemic resistance (2,87,91). A role for induced resistance in the biological control of *Fusarium* wilt of carnation (*Dianthus caryophyllus*) by *Pseudomonas* spp. strain WCS417 was demonstrated by spatial separation of pathogen and inducer strain (87). Carnation roots were treated with the bacterium prior to inoculation of stem tissue with *Fusarium oxysporum* f. sp. *dianthi*. Uncharacterized signal molecules produced by strain WCS417 in the roots induced phytoalexin accumulation in the carnation foliage. Phytoalexins were produced if pathogen introduction followed bacterial application but not by bacterial treatment alone (86). Alstrom (2) reported that bacterization of bean seeds with *P. fluorescens* strain S97 reduced the numbers of foliar lesions due to the halo blight pathogen, *Pseudomonas syringae* pv. *phaseolicola*. The systemic resistance response was eliminated upon autoclaving of the bacterial suspension, suggesting S97 secreted substances during seed germination which were translocated to the foliage. Strains active in PGPR have been investigated as inducing agents of systemic resistance for the *Colletotrichum orbiculare*-

cucumber host-pathogen system (91,92). Bacterial strains applied to cucumber seed were not recovered from cucumber petioles, suggesting induced systemic resistance was a mechanism rather than competition or antibiotic production (91,92).

Uncharacterized Biological Control Mechanisms

In many recent efforts, biological control studies using pseudomonads have evolved from descriptive studies to a more mechanistic approach. Many of these host/pathogen/biocontrol systems have been characterized at the genetic level although the exact nature of the antifungal compound has yet to be identified (25,28,38,71,72,80).

Unidentified antifungal metabolites of *P. fluorescens* HV37a, one of which has been recently termed oomycin A (37), reduced the incidence of preemergence damping-off of cotton by *P. ultimum* (38). A 70% reduction in root infection and a 50% increase in seedling emergence were attributed specifically to oomycin A production. This compound remains structurally uncharacterized. An iron-antagonized inhibitor (FAI) produced by *Pseudomonas* spp. strain NZ130, with a fungistatic mode of action against *P. ultimum* was analyzed using isogenic mutants and a β-galactosidase reporter system (25). The reversible nature of inhibition by FAI with Fe^{+3} concentrations greater than 10 μM suggested deprivation of an essential nutrient as the antifungal mechanism.

Genetic analysis of 48 *P. fluorescens* mutants altered in repression of the take-all pathogen provided evidence in support of multiple genes with an active

function for expression of antibiosis (71). Further characterization of mutants and wild-type strains in growth chamber studies indicated a lack of correlation between the level of antibiosis on PDA and the level of take-all pathogen suppression (72). Although the exact nature of the antifungal activity was not identified, surprisingly, mutants for increased antibiosis *in vitro* produced higher disease ratings than the parental strain when tested *in situ*.

Sugimoto et al. (80) attempted to exploit indigenous compost-inhabiting oligotrophic bacteria (45% of the 138 isolates were subsequently identified as *Pseudomonas* species), using *in vitro* inhibition bioassays and *in situ* studies employing bark compost medium. The majority of the 138 isolates tested were classified as facultative oligotrophs that produced unidentified antifungal compounds with activity against *P. ultimum*.

Studies were conducted at six locations across three states over two field seasons to evaluate selected *Pseudomonas* biocontrol strains for suppression of *P. ultimum* and *R. solani* cotton seedling disease (28). The results indicated that several strains improved seedling stands at multiple locations; stand counts were increased following granular and liquid in-furrow application methods but not following seed application.

Although fluorescent soilborne pseudomonads possess an arsenal of antifungal inhibitory mechanisms, it is unlikely that a single strain will be capable of broad spectrum biological control. Rather, successful control of a wide range of soilborne pathogens may best be achieved though the use of a mixture of

pseudomonad strains, each of which has been genetically modified for optimum biological activity of a single mechanism under a defined and unique set of environmental conditions. These strains introduced collectively, may achieve biological control under variable environmental conditions, the situation most likely encountered in the field situation. Accomplishment of this approach will require additional characterization of individual antifungal mechanisms.

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III.

A COMPUTERIZED IMAGE ANALYZER PROGRAM FOR AGRICULTURAL APPLICATIONS[1]

Abstract

A computer software program, TIFF (Tag Image File Format) Image Analyzer or TIA was developed that utilizes the TIFF format to accumulate information on root and foliar samples placed directly on a desk-top scanner. Accumulated data are stored as an ASCII file that can be imported and subsequently manipulated using spreadsheet and statistical software packages. Applications of the program could include the study of seasonal leaf area development, crop foliar yields, pesticide spray drift patterns, root surface areas, and total root area reductions/losses due to root disease pathogens.

Introduction

A multitude of computer software programs are being continuously developed to increase the speed with which researchers can accomplish such tasks as data acquisition, processing, and graphic presentation. In addition to computers, desk-top scanners and associated software have become valuable tools that allow the user to incorporate both images and text directly into most word processing

[1]Technical details regarding software development were contributed by Mark Piermarini, former student in the Department of Electrical Engineering, VPI & SU

programs without major modifications. The TIA program was developed as a software interface between two basic pieces of office/laboratory equipment, a computer and desk-top scanner, to express the dynamics of root colonization by biological control organisms. By means of this program, colonization can be assessed as a function of available root surface area rather than the more conventional root length or root weight. Davies and Whitbread (1) compared procedures to find a rapid and sensitive method for estimating root surface area and concluded that root dry weight provided the best estimate. True root surface area can be estimated by the product of π and the projected surface area, and varies linearly as a function of root diameter. In contrast, root weight is related to volume and varies as the square of the radius, assuming a uniform density of root volume. As roots mature and differentiate, their diameter increases, invalidating the assumption of uniform density along a root length. Populations expressed relative to surface area account for changes in root diameter and are therefore more representative of the actual area available for colonization.

A significant problem in monitoring and comparing root populations within a single plant or between plants is the inherent variability involved. This variability can be diminished by sampling a large number of root systems, a time consuming and tedious task. The TIA software program overcomes this problem by allowing rapid and efficient processing of large numbers of root samples. In addition, the ability to calculate the surface area of photographs of roots and foliage, eliminates the necessity for immediate scanning to avoid sample deterioration. Optionally,

Microsoft Windows™ provides an interface between TIA and any number of scanning software packages chosen by the user, reducing the time to seconds between image processing and analysis. Since area calculations of objects with irregular boundaries can be laborious and highly inaccurate, the program proved useful for agricultural applications other than root surface area. An abstract of this information has been published previously (2).

Basic Hardware and Software Requirements

The computer system and desk-top scanner used for data acquisition was an Apache 386 and a Hewlett Packard (HP) ScanJet IIP respectively, although most other systems will suffice. Minimum computer requirements include an IBM PC 8086, or compatible, with 410K free memory and a VGA adapter card. However, for optimum image processing speed, a 386 or higher is most desirable. Many desk-top scanners such as the Hewlett Packard (HP) ScanJet IIP are bundled with their own Microsoft™ digitizing software. Image files created using scanning software can be analyzed directly with the TIA program or images can be enhanced using Photofinish™, an accessory to the ScanJet basic software which contains a wide variety of image processing features. Depending on personal preference, Image-In Scan and Paint (CPI S.A., Geneva, Switzerland), is another versatile image digitizing software package that can be utilized. Using either of the above programs, images are scanned as a drawing or photograph image type, and saved as TIFF files, one of many standard image file formats. Once TIFF files are created and stored, the TIA program is used for image recognition and analysis.

Image Recognition and Analysis

TIFF version 5.0 description

The image recognition and analysis code requires a description of the TIFF version 5.0 specification to determine the characteristics of an image. A TIFF consists of four main sections: header, directory of tags, actual tags, and bit-map image data. This specification is based on the fact that a group of fields, called tags, describes an image. Tags describe characteristics of an image such as its width, length, compression type, number of bits of resolution per pixel, and samples per pixel. An image requires some basic tags to be considered valid while others are optional. For more information see the document, "Tag Image File Format Specification Revision 5.0 Final" (Microsoft™ and Aldus Corporations).

TIFF structures and tag handling

The first requirement in image analysis is to determine if the image conforms to the TIFF standard, followed by reading the tags and processing the information obtained from the image. An initial default image descriptor is built, which has specific tags set to default values (as defined in the TIFF definition). If one of the more than 45 tags is found in the TIFF, it will override the default values. Images that do not override default values are processed as if they were created with the defaults, whereas tags that do not affect the image are ignored.

Image analysis and data compression

Images with 4 bits of resolution per pixel can contain 16 distinct values, and images with 8 bits pixel resolution can have 256 values. If the data are

compressed black and white images, using the only type of compression currently supported (CCITTGroup 3, International Telegraph & Telephone Consultative Committee), the image is first decompressed. This method compresses bi-level images into a series of alternating lengths of either white or black data. Throughout the data processing phase of any image, the program accumulates the number of times each distinct pixel occurs for histogram formation and later computations.

User Interface, Databases and Image Modification

Overall description

Convenient mouse pulldown menus allow the user to interface to the Paradox® database format (version 3.5, Borland International, Scotts Valley, CA) to store image data and statistics. The Paradox® database format is widely used and is an industry standard. If the user does not wish to use Paradox®, TIA in turn can export the data to an ASCII file and add it to any number of database fields the user chooses. ASCII files can be imported into nearly all spreadsheet and numerical analysis programs currently available for data manipulation or graphic presentation.

The program prompts the user for two values, detailed below, each time it is accessed. Changes in these two values affect images analyzed from then on; no recalculation of image area is performed on images already stored in the database. The first value is the 'image reduction/enlargement factor' which is the percentage the image in a photograph was reduced or enlarged. The default value of 100%

indicates the original size. The second prompt is the 'selection point' that the program uses to compute area. The selection point of an image is defined as the value (a number between 0 and 256) on the histogram plot where all pixel values equal to and below this point are changed to black (actual image being scanned) while those above this point are changed to white (image background). The default or initial selection point for the histogram is "0", meaning only the true pixel value for solid black would be considered as an area value. The default value that appears may be altered and stored for area calculation of many replicate samples. Depending on the scanner model, the pixel value "zero" is considered as black, whereas, other scanners perceive it as white. The TIA program recognizes the '0 as black' convention, so images processed using the other notation are converted before analysis. The majority of scanning software packages contain a "reverse" option which inverts the image dots creating a "negative" appearing image. For a gray scale image, each hue is changed to its complement, i.e., a 30% gray color will change to a 70% gray. If root systems or other white objects are scanned using the 'bi-level (black and white) drawing' option, the "reverse" command is employed. Roots or images scanned using the 'photo image' option will produce a frequency histogram precluding the need for inversion.

Database definition and functions

The TIA program prompts the user for the database of records that will be used to add new images. Alternatively, one can be created if none exist. This convention allows multiple users to maintain separate data files since any user can

navigate through the directory structure to find an existing database file. The database consists of the following fields or information; 1) photograph or image number, 2) date image was scanned, 3) age of the image (if applicable), 4) image width and length (cm), 5) X/Y axis resolution (dots per unit length), 6) area (cm^2), precision to five significant digits), 7) number of gray scale levels, 8) selection point on the histogram, and 9) image description. These fields are saved should it be necessary to re-scan and analyze a particular image. Once an image is analyzed and/or modified, it can be saved to the database. The program prompts the user for information about the image, such as the photograph/image number, image age, and a brief file description prior to saving it. The scan date is set to the current date (obtained from the computer) but can be changed. These same prompts are presented if the user is modifying an existing database record, possibly due to a data entry error. Under the "Database Utilities" pulldown menu, records can be viewed, deleted and printed. The database and all associated files can also be copied for backup purposes.

File selection and gathering statistics

From within the "Process Image" pulldown menu a user can select a TIFF file in the same manner as the database file was selected at the start of the program. A "percentage completed" bar is drawn on screen to indicate how far the data gathering has progressed. This menu also contains an image modification option, taking into account image coloration or shades of gray.

Image modification

Once a TIFF file is retrieved, a histogram of the pixel value versus color frequency is plotted, if an image has been scanned as a photograph image type, *e.g.*, for images containing many shades of gray or color. The data represented on the graph are the pixel frequency values of the image prior to area calculation. The 'selection point' and the 'image reduction' factor are used in calculating the image area. As mentioned above, the initial selection point and area calculation that appears is "0" for the frequency plot of the raw data. Executing <Control + F2>, initiates the user-stored selection point to determine an area value. If the user-stored selection value is greater than the number of distinct pixel values in the current image, the program prints an error message and pauses until the user changes the value to within the image range. The user may alter the selection point, and thus the area, based on the histogram pixel distribution, or if selected portions of the image are of interest. <Control + F1> restores the original raw data histogram. Images saved as bi-level produce a bar graph frequency of either "0" (black) or "1" (white) pixels only, the area value produced corresponding to the frequency of black pixels (image area). Images scanned in inches are converted to cm prior to area calculation.

Miscellaneous options and error messages

An additional "Utilities" pulldown menu allows the user to move from the program shell to DOS and execute any valid DOS command from within the program. There is also an image summary option that displays the current image

statistics. The number of records in the database is shown at the bottom of the screen, along with the current date and time. Two particularly valuable assets in the program include a help menu and an on-screen error display should errors occur in the TIFF analysis code or while executing menu functions. If a database or menu error occurs, it is advisable to save the current image record, if one exists, and exit the program. Errors returned from the TIFF analysis code usually indicate that the image cannot be analyzed with the program.

Image Scanning Optimization and TIA Calibration

A Thickness Contact Reticle Gauge, 2.7 cm total diameter, with 13 different pigment-filled glass etched rectangles ranging in width from 0.004 to 0.04 cm was purchased from Edmund Scientific Co. (Barrington, NJ), to calibrate and determine the accuracy limits of the TIA program (Fig. 1). The resolving power of the program at 300 dpi was 0.004 cm width. Utilizing any one of several editing features within the scanning software, (e.g., touch-up and/or a higher scanning dpi), the resolution can be further enhanced. However, for each resolution doubling, the resulting image file size quadruples. A series of fishing line segments, simulating root pieces of various widths, were coated with white latex paint and scanned to determine the accuracy in measuring 3D object areas. The ScanJet's scanning software contains an automatic adjust icon for optimizing the highlight, shadow, contrast and brightness settings for the image, all of which may be independently manipulated manually as well by experienced users. The automatic exposure settings selected by the scanning program produced images

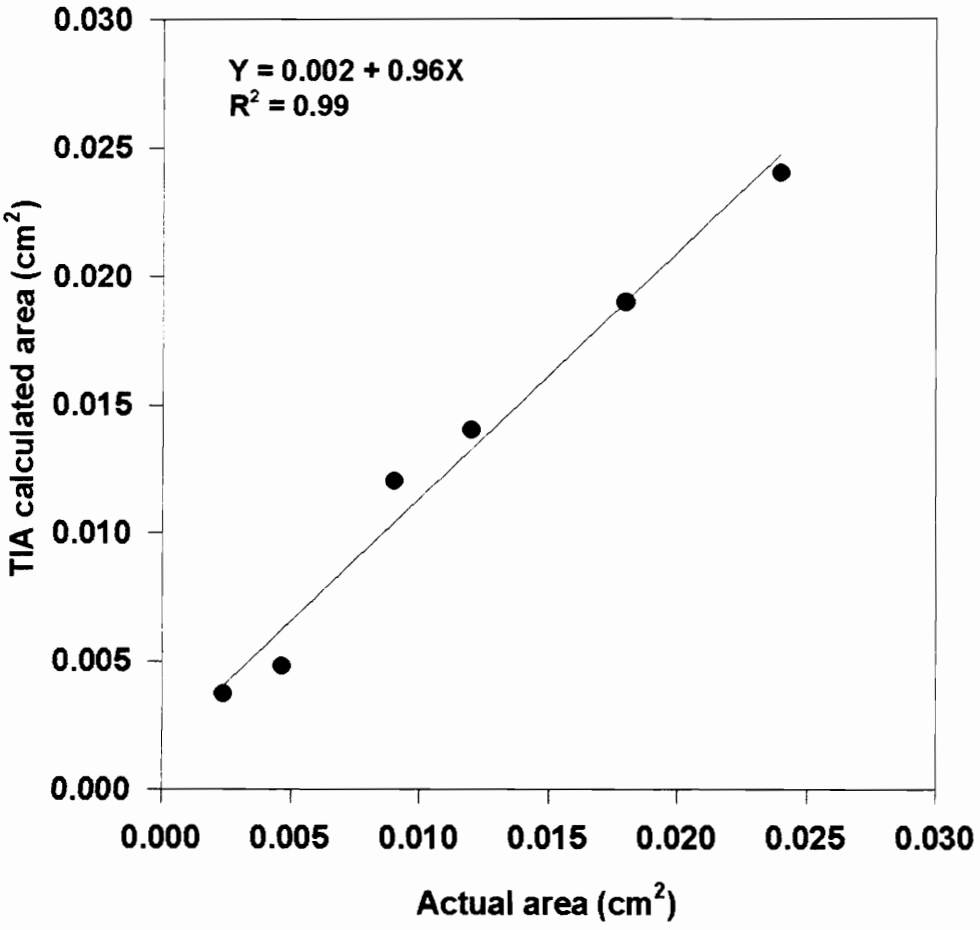

Figure 1. Comparison of actual and TIA calculated area of pigment-filled glass etched rectangles of similar length but varying width (cm) on a Thickness Contact Reticle Gauge. Width values ranged from 0.04 to 0.004 cm. Actual area (cm^2) was the product of length and width, whereas, TIA area was determined from the scanned image.

which provided accurate TIA area calculations for line segments greater than 0.198 cm in diameter, but slightly underestimated values below this range (Fig. 2). For line diameters smaller than 0.05 cm, the automatic exposure settings produced slightly overestimated area values. The most accurate area determination using the auto settings occurred when several line diameters were scanned simultaneously, the situation most likely to occur when scanning plant root systems. Calculated projected area for all segments was 3.36 cm^2, compared to the TIA value of 3.41 cm^2, an error of 1.49%.

Additional calibration and resolution was performed as described above for the thickness gauge using a MM Square Contact Reticle Gauge consisting of a 1 cm square subdivided into a series of smaller squares. The resolving threshold area value was 0.008 by 0.008 cm (0.000064 cm^2), the smallest value on the reticle gauge, using the scanning conditions described above. The results (Fig. 3) showed a precise correlation between actual and TIA calculated area as indicated by the high R^2 value. Smaller area determinations may theoretically be possible, as indicated above.

Agricultural Applications

Applications relevant to agriculture may include the study of leaf area development, the assessment of foliar reduction due to insects, air pollutants, drought or disease, crop foliar yield analysis, and estimation of plant root surface area and reductions in total root area in response to root rotting soilborne pathogens.

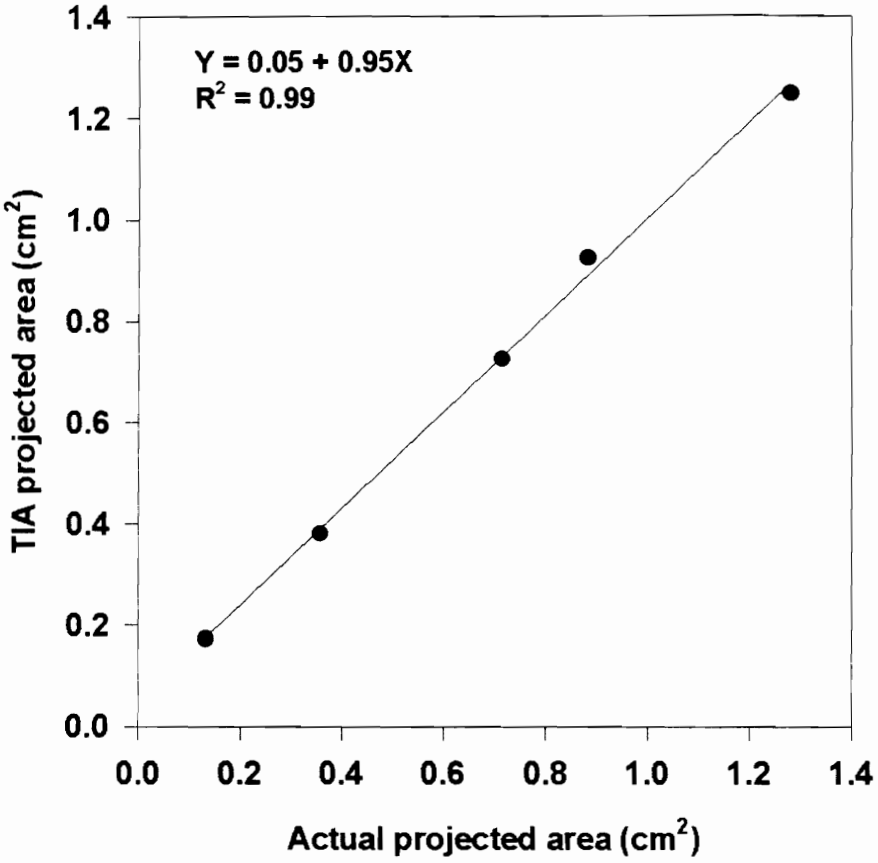

Figure 2. Calibration of the TIA program using latex-coated fishing line of various diameters for root measurement. Line diameter, determined by a microcaliper, varied from 0.02 to 0.198 cm. TIA projected area was estimated from the scanned image.

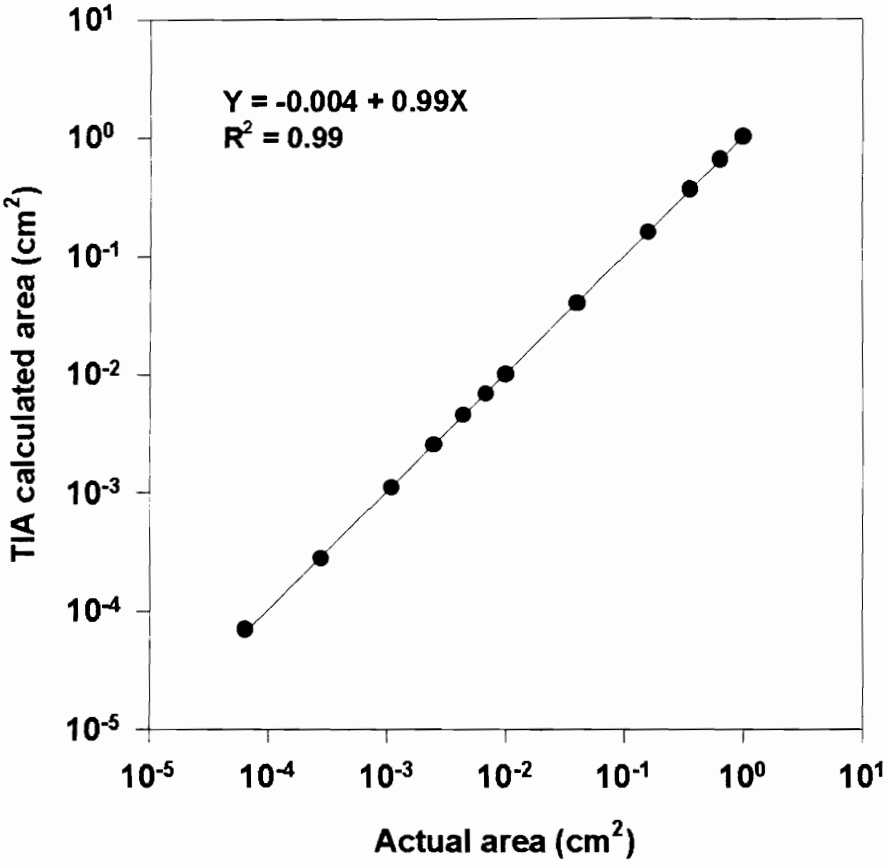

Figure 3. Comparison of actual and TIA calculated area of a 1 cm square and smaller square subdivisions of known area on a glass etched MM Square Contact Reticle Gauge.

Corn root area

Corn (*Zea mays*) roots of relatively constant lengths (±1 cm) but varying diameters were scanned using manually optimized, i.e., fine-tuned automatic exposure settings (Fig. 4). Overall, TIA projected area values differed from microscopically determined values by 3.3%. The difference between calculated and TIA projected values using auto versus manually optimized settings was 5.0 and 3.3%, respectively. Assuming the majority of plant roots to be cylindrical in shape, multiplication by π would convert projected area to total surface area. Manually adjusted values require a greater initial time input; however, once settings are optimized and saved as a template file, samples can be analyzed in rapid succession. For plant treatment comparisons, optimum accuracy is achieved through maintenance of a constant scanning selection area (height and width, cm) for all samples within an experiment. The number of samples processed using either manual or automatic exposure settings is limited only by the maximum scanning area dimension (21.5 x 30.0 cm for the Scanjet IIP).

Leaf area

Area calculations can be attained for any size, number, or shape of objects placed within the scanning field. Foliage from an entire plant can be scanned in a single run, depending on individual leaf size, eliminating the need to feed individual leaves by hand onto a conveyor belt (LiCor® leaf area meter). The TIA program can determine the area of both small and large leaves, including thin grasses and conifer needles, with equal accuracy (Table 1). The scanning

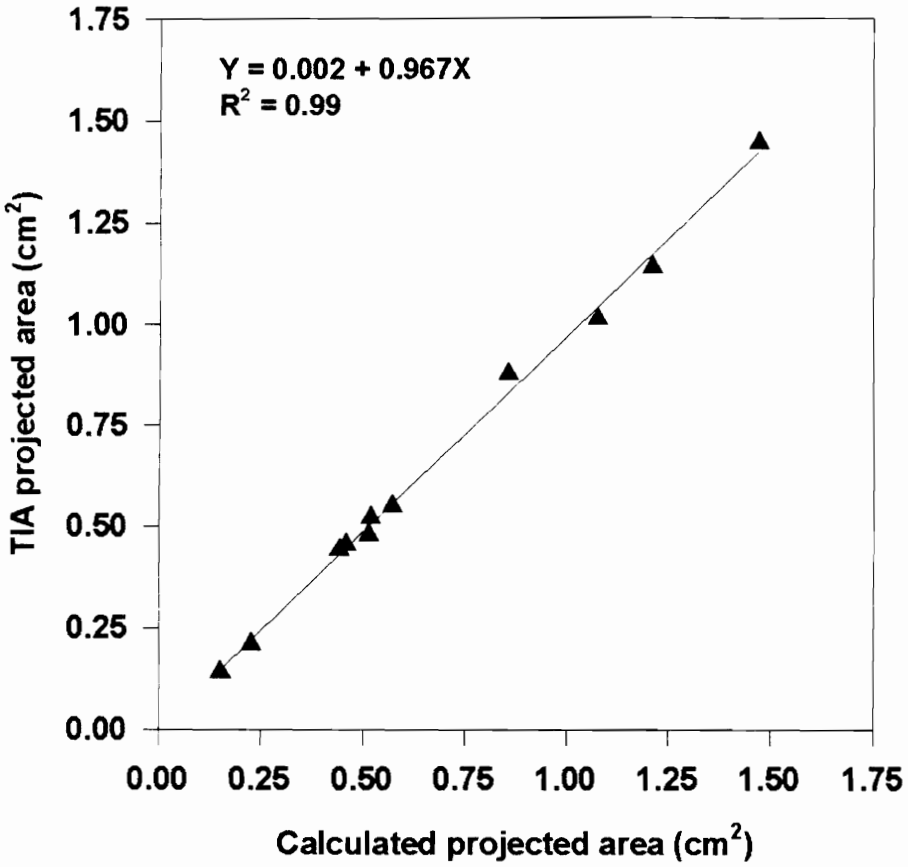

Figure 4. Comparison of projected area of 11 different diameter corn roots of similar length (±1 cm). The TIA area was estimated from the scanned image, whereas calculated area was estimated from microscopic measurements of root diameter.

Table 1. Comparison of the LiCor leaf area meter and the TIA software program for area calculation of various leaf shapes and sizes.

	Area (cm^2)[1]	
Leaf species and margin type	LiCor	TIA
Simple leaf, bristle-tipped teeth [Pin Oak (*Quercus palustris*); 4 leaves]	90.77 ± 0.07	92.34 ± 0.05
Compound leaf, entire margin [Black locust; *Robinia pseudoacacia*); 17 leaflets]	185.01± 0.08	184.51 ±0.05
Grass species [Johnson grass (*Sorghum halepense*); 1 leaf]	21.58 ± 0.23	21.68 ± 0.05
Simple leaf, large toothed margin [American chestnut (*Castanea dentata*); 1 leaf]	66.50 ± 0.10	67.30 ± 0.05
Compound leaf, finely serrated [Trumpet-creeper (*Campsis radicans)*; 5 leaflets)	67.35 ± 0.03	68.21 ± 0.05
Conifer needles [White pine (*Pinus strobus*); 3 needles]	1.89 ± 0.03	2.26 ± 0.02

[1]Each leaf, leaflet or needle measurement repeated four times.

program detected even narrow serrations or needle-like margins, hence the slightly larger area values for the TIA program.

Loss of root area due to soilborne pathogens

The total root area for a representative cotton (*Gossypium hirsutum*) plant grown in soil containing no seedling disease pathogens was calculated as 22.06 cm^2 (Figure 5). In comparison, the root area for a cotton plant grown in soil with high levels of *Pythium ultimum* and *Rhizoctonia solani* propagules was 5.32 cm^2 (Figure 6), demonstrating the extent of root loss under severe pathogen pressure.

Scanned image files can be stored on either a hard drive or diskettes as a permanent record and for future image manipulations. A study involving spray drift area accumulation on dye-impregnated water-sensitive cards (7.6 x 12.7 cm) provided useful data on pesticide drift patterns under various simulated wind conditions (unpublished data). An added feature of this program lies in the fact that root systems (or other images) can be photographed using Ektar 125 color print film, or T-max 100 black and white film and scanned at a later date, eliminating the problem of desiccation or decomposition that occurs when processing large numbers of samples. The TIA program has a built-in percentage increase or reduction factor for area calculations of photographic images less than or greater than actual size, respectively. The photo then provides a permanent record of the image should re-scanning become necessary. Image statistics as noted above include photo number if this option is selected. Investing a small amount of time and patience learning to use any of several image scanning

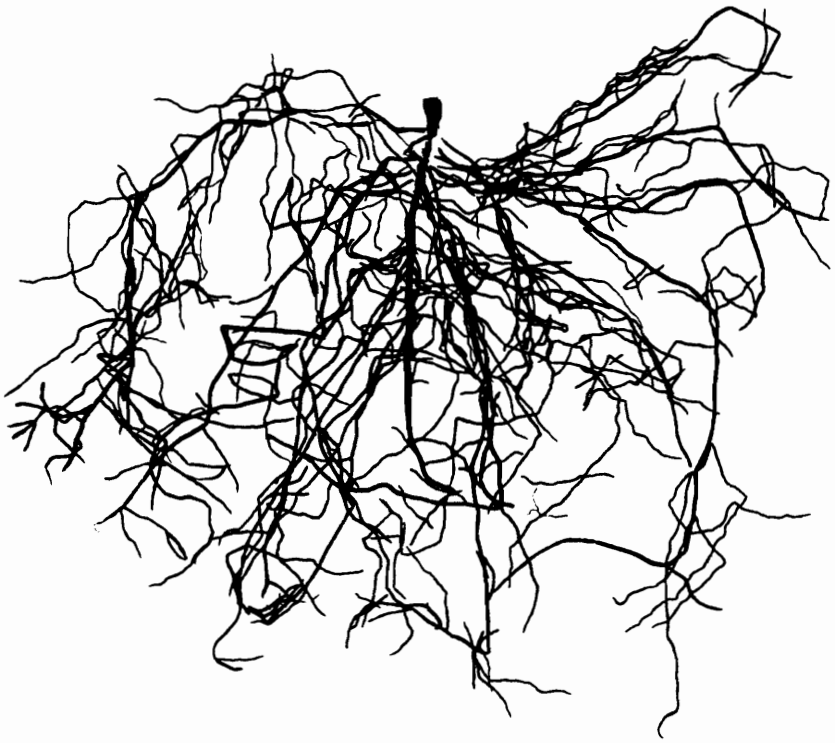

Figure 5. Scanned image of healthy cotton root system, total root area, 22.06 cm^2, from plants grown in soil without *Pythium ultimum* and *Rhizoctonia solani* propagules.

Figure 6. Scanned image of cotton root system, total root area 5.32 cm^2, from plants grown in soil infested with high inoculum densities of *Pythium ultimum* and *Rhizoctonia solani*.

software, will enable the user to obtain rapid, accurate, and reproducible estimates of image surface area using the TIA program.

Literature Cited

1. Davies, K.G. and Whitbread, R. 1989. A comparison of methods for measuring the colonization of a root system by fluorescent pseudomonads. Plant and Soil 116:239-246.

2. Mulesky, M. Chism, B., Piermarini, M., and Hagedorn, C. 1992. A computerized image analysis program for agricultural applications. (Abstr.) Phytopathology 82:1155.

IV.

COLONIZATION STUDIES OF COTTON ROOTS BY *PSEUDOMONAS CHLORORAPHIS* ANTIBIOTIC AND SIDEROPHORE MUTANTS

Abstract

Cotton seedling disease caused by *Pythium ultimum* and *Rhizoctonia solani* occurs worldwide in soils varying in pH from 4.5 to 8.5. Biocontrol of these pathogens should include bacterial isolates capable of colonizing cotton roots within this pH range. *Pseudomonas chlororaphis* L-850, a strain that produces siderophores and antifungal antibiotics, and two Tn*5* insertion mutants of L-850 lacking siderophore (*sid*) and antibiotic (*ant*) activities were evaluated in the greenhouse in two pathogen-free soils (Suffolk sandy loam, pH 5.7 and the other a Ross loamy sand, pH 8.0). Plants in Conetainers® were embedded in moist sand to assess bacterial movement following seed application in the absence of downward water percolation. Root sections (upper and lower tap and upper and lower laterals) were sampled 22, 36, and 50 days after planting (DAP). Mutant and wild-type strains migrated along the root surface as roots elongated but varied in population density. Slight increases in population density occurred on upper tap and upper lateral roots between day 22 and 36 in both soils. Mean populations of bacteria ranged from log 6.0 cfu/cm^2 root surface area on the upper tap roots to log 4.6 cfu/cm^2 on the lower laterals. The absence of siderophore biosynthesis

appeared to enhance the rhizosphere competence of *P. chlororaphis*. Moreover, the ability of the *(sid)* mutant to maintain a more uniform root distribution pattern of higher population densities than either the wild-type or *(ant)* mutant may have contributed to the significant root growth promotion observed.

Introduction

Among the diverse genera of bacteria occupying the rhizoplane/rhizosphere environment, fluorescent pseudomonads have been most frequently associated with growth promotion and the suppression of many soilborne pathogens of agronomic importance (3, 23, 26, 27, 28, 42, 45). In cotton, the seedling disease fungal pathogens *Pythium ultimum* and *Rhizoctonia solani*, are responsible for substantial annual stand and yield losses, with estimates ranging from 1.0 to 6.5% annually (6, 9). The disease is of major importance not only in the U.S. but worldwide. Cotton and its fungal pathogens tolerate a fairly wide range of soil acidity and alkalinity with soil pH's of 8.0 to 8.5 in cotton growing regions of Africa, to values of 4.0 and 5.0 in regions of Indonesia (35).

Colonization of the host plant rhizosphere is of primary importance in suppression of soilborne plant pathogens by introduced bacterial biocontrol strains. The effects of soil moisture on root colonization of seed- or furrow-applied pseudomonads has been the subject of several investigations (2, 17, 29, 38). Spatial colonization patterns have emphasized the importance of vertical soil water movement on population distribution along root surfaces after irrigation (2, 13, 29,

38). Adequate soil moisture alone did not appear sufficient to establish high bacterial populations in pathogen infection sites (13). Wheat (*Triticum aestivum*) root colonization by a fluorescent *Pseudomonas* strain was postulated to occur by passive transport on the root apex followed by multiplication on the root surface once cell deposition occurred (21).

Therefore, for effective biocontrol of these pathogens by pseudomonads, preliminary screening bioassays should focus on those strains capable of proliferation and population persistence at levels sufficient to provide initial short-term control of the pre- and postemergence damping-off that results in premature seedling mortality (phase 1). Subsequently, introduced bacteria should also provide extended protection against the hypocotyl/root rot and lesion induction on surviving seedlings and more mature plants (12) (phase 2), that can substantially reduce the root surface area available for water and nutrient uptake throughout the growing season.

Twenty pseudomonad isolates provided though cooperative agreements between VPI & SU and Ecogen Inc., and the former Crop Biotechnology program within Allied Corporation, demonstrated acceptable biocontrol activity in studies involving cotton seedling damping-off by *P.ultimum* and *R. solani*. These isolates also produce siderophores and one or more antifungal antibiotics. The objectives of this study were to: i) screen the isolates for their potential to extensively colonize cotton roots in the absence of vertical water percolation; ii) select a single strain capable of colonizing cotton roots in two soil types at two pH extremes (pH 5.7,

8.0) to provide suppression of these pathogens across a wide range of soil pH; and iii) compare antibiotic-negative (*ant*) and siderophore-negative (*sid*) derivatives of the wild-type strain selected in objectives i and ii, for differences in population density and distribution patterns on cotton roots. A preliminary report has been published (37).

Materials and Methods

Bacterial strains, fungi, media and growth conditions. The 20 pseudomonad biocontrol strains and their origins are listed in Table 1. Media used for antifungal bioassays and greenhouse colonization studies included King's medium B (KB) (24) and modified RSM (mRSM). RSM is a medium developed by Buyer et al. (11) modified to contain 1% glucose, 0.5% casamino acids, 1.56 µM $MnSO_4 \cdot H_2O$, and 100 µM Fe^{+3} supplied as diethylenetriamine pentaacetic acid (DTPA), when necessary, for inhibition/promotion of siderophore/antibiotic biosynthesis. For DNA manipulations and transformation procedures, *Escherichia coli* and pseudomonads were grown in Luria Bertani (LB) (40), and Tryptic soy broth (TSB; Difco, Detroit MI), respectively. *Escherichia coli* and pseudomonad incubation temperatures for all experiments were 37 and 28 C, respectively. Turbidity (Beckman model DU-64 Spectrophotometer) measurements for broth shake cultures were performed at 640 nm (A_{640}). Auxotrophic mutants of putative transconjugants were detected on Davis' Minimal medium (14) amended with kanamycin (DMM_K) and discarded. Antibiotics, when necessary, were added at the following concentrations (µg/ml):

Table 1. Strain designation, culture characteristics and source of *Pseudomonas* spp.

Strain	Fluorescence[1]	Pigment[2]	Source
L-849	Y	N[3]	Ecogen
L-850	Y	Orange	Ecogen
L-851	Y	Orange	Ecogen
L-852	Y	Orange	Ecogen
L-853	Y	Orange	Ecogen
L-854	Y	Orange	Ecogen
L-855	Y	Orange	Ecogen
L-856	Y	Orange	Ecogen
L-886	Y	N	Ecogen
L-890	Y	N	Ecogen
L-892	Y	Orange	Ecogen
G226	Y	N	Allied
M-17	N	Brown	Allied
AD4-34[4]	Y	Yellow	Allied
TR-21	Y	Orange	Allied
Ral-3	Y	N	Allied
WSB15134	Y	N	Allied
31-12	Y	N	Allied
AC4-52	Y	Violet	Allied

[1] Fluorescent pigment production at 366 nm wavelength.
[2] Production on modified RSM medium + 100 µM Fe^{+3} (see text pg. 4).
[3] N = None.
[4] Uncertain identification as *Pseudomonas* spp.

trimethoprim (Tp), 200; kanamycin (K), 50; nalidixic acid (N), 20-200; and rifampicin (R), 20-200. Generation times (g) were calculated for the mutants using the equation [g = Time (t_1 - t_o) (h) x (log 2/$\log_{10}N_1$ - $\log_{10}N_0$) where N_1 and N_0 are the number of cells/ml at time t_1 and t_0]. The fungi *P.ultimum* and *R. solani* were grown on Potato dextrose agar (PDA; Difco, Detroit, MI) for 7 days prior to *in vitro* bioassays.

Selection of twenty spontaneous antibiotic resistant mutants. Stock cultures of the 20 pseudomonad isolates were streaked onto KB and incubated overnight. Single colonies were selected for each isolate and suspended in 50 ml KB. Cultures were placed in a shaker-incubator (120 rpm) until mid-log phase (turbidity of 0.4 at A_{640nm}) was reached. Aliquots (50 µl) were spread over the surface of 10 KB plates amended with 20-200 µl/L R and N. Plates were incubated until colonies were visible (24-36 h). Putative resistant colonies of each isolate were tested by restreaking on antibiotic amended medium. Stock cultures of mutants were stored at -80 C in 15% (v/v) glycerol.

Preliminary screen for cotton root colonizing strains. Antibiotic resistant mutants were streaked from stock cultures (-80 C) onto KB supplemented with 80 and 160 µg/ml N and R (KB_{NR}), respectively. Single colonies of each isolate were placed in 50 ml KB_{NR} broth until a viable cell density of 10^8/ml was attained, as determined from regression plots of 20 individual growth curves. Cells were pelleted by centrifugation, washed once with 0.1 M phosphate buffer (pH 7.0) and resuspended in 50 ml 1.5% carboxymethyl cellulose (CMC). Surface sterilized

(1.7% sodium hypochlorite, 5 min), acid-delinted cotton seed 'Deltapine 50', were placed in 50 ml conical centrifuge tubes containing the bacterial suspension at a density of 20 seeds/30 ml. The contents of each tube were vortexed for 5 min to ensure even distribution. Treated seeds were dried in ultraviolet (UV)- sterilized plastic weigh boats for 60 min in a laminar flow hood. Mean number of cfu was 10^8/seed as determined by dilution plating of 4 replicates (seeds) on KB_{NR}.

Conetainers® (Ray Leach Conetainers®; Portland, OR), 20.9 cm high and 3.8 cm wide were filled to within 3 cm of the top with a 1:1 (v/v) mix of sterile sand-vermiculite, submerged in water to rim level, and allowed to drain to field capacity. Two treated seeds per Conetainer® were covered with 1.8 cm of moistened mix. Controls consisted of 1.5%-CMC-treated or nontreated seeds. Conetainers® were placed in trays and the trays loosely sealed in plastic bags to maintain soil moisture at or near field capacity. Greenhouse temperatures were held at a 24/21 C day (14 h)/night (10 h) cycle. Plants were harvested 14 days after planting (DAP) during which time they received no additional water or light. Conetainers® were sliced in half longitudinally with a scalpel and seedling root systems gently teased from the surrounding medium. After gentle shaking to remove all but the tightly adhering rhizosphere soil, root systems were divided into 1.5 cm segments with a sterile scalpel and assayed on KB_{NR} for extent of colonization using an agar imprint technique. Root segments were pressed into the agar on plates subdivided and numbered from 1-6 with 1 being the seed coat if present, and 2-6, 1.5 cm incremental sections from hypocotyl to root tip. Root segments were retrieved

from the agar after 60 min incubation and plates further incubated an additional 36 h. The extent of bacterial growth in each numbered section was scored visually using a (-) (no growth) and a (+) to (++++) rating system with a single (+) representing a small amount of bacterial growth and four (++++), profuse growth within the entire imprint trough.

Transposon mutagenesis, DNA isolation and southern analysis. The mini-Tn*5* transposon system developed by de Lorenzo et al. (15) was used for insertional mutagenesis of pseudomonads to generate mutants deficient in antifungal (antibiotic) activity. Protocols for optimization of transformation and conjugation were provided with the transposon system. The plasmid maintenance strain *E. coli* CC118 (λ*pir*) and the untransformed donor strain *E. coli* S17-1 supplied with the transposon system were incubated in media containing Tp and K respectively, for all experimental protocols. Plasmid pUT containing the mini-Tn*5* element was isolated from *E. coli* CC118 and purified using protocols accompanying the Insta-Prep kit (5 Prime → 3 Prime Inc.; Boulder, CO). Preparation of $CaCl_2$-induced competent cells of the donor strain *E. coli* S17-1 (41) and subsequent transformation of S17-1 with the pUT plasmid was performed as described (31). Confirmation of plasmid uptake in S17-1 was carried out as for *E. coli* CC118. *E. coli* donor and pseudomonad recipient cells were incubated overnight in 30 ml LB_K or TSB, respectively, until a turbidity of 0.12 at (A_{640nm}) for each was obtained. A 1:1 mixture of donor and recipient broth suspensions was impacted by suction onto a 0.22 μ Supor 200® (Fisher Scientific; Norcross, GA) filter and washed twice with

10 mM $MgSO_4$, prior to incubation of the filter mating mixture on LB plates for 17.5 h. Filter contents were suspended in 10 mM $MgSO_4$ and 50 µl aliquots spread onto KB_{KN} to select for recipient strains and to counterselect against the donor strain. After a 24 hour incubation, transconjugant colonies were transferred to fresh KB_{KN} with toothpicks followed by replica plating onto DMM_K for identification and subsequent elimination of auxotrophic mutants.

Restriction enzyme digests of plasmid and chromosomal DNA were performed as recommended by the supplier (United States Biochemical; Cleveland, OH). The pUT plasmid was digested with *Eco*RI and run on a 0.8% agarose gel to electrophoretically generate the intact Tn*5* element for use as a genomic DNA probe. The GlassMax™ DNA Isolation Matrix System (GibCo BRL; Gaithersburg, MD) was used as per manufacturers instructions for extraction and purification of the 2.0 kb *Eco*RI fragment from the agarose for probe synthesis. Nick translation was performed according to instructions accompanying the Rad Prime DNA Labeling System (GibCo BRL; Gaithersburg, MD). Chromosomal DNA from pseudomonads was isolated using a protocol modified (Appendix A) from that developed by Marmur (33). Genomic DNA transfer to Magna nylon membranes following electrophoretic separation, and probe hybridization using high stringency conditions were carried out according to manufacturers recommendations (Micron Separations Inc., Westboro, MA). Transconjugants were initially screened for loss of antibiotic activity by stab-inoculating 10 strains around the perimeter of an mRSM plate supplemented with 100 µM Fe^{+3} (to inhibit siderophore production).

Plates were incubated for 24 h prior to challenge inoculation in the center of the plate with a 4 mm diameter plug of *P. ultimum* or *R. solani.* Plugs were taken from 7-day old mycelial colonies growing on PDA. Presence or absence of zones of inhibition was recorded after an additional 24 h incubation. Transconjugants with no measurable inhibition zones were further assayed using two antifungal agar diffusion techniques. The first technique involved growing each isolate overnight in mRSM-Fe_K broth, with shaking (120 rpm) to a standard turbidity of 0.7 at A_{640nm} (10^9 cells/ml). Aliquots (400 µl) were spread over the surface of a 0.2 µ pore size Supor 200 membrane filter (47 mm) placed in the center of a mRSM-Fe_K agar plate. Filter suspensions were incubated for 24 h, and subsequently removed from the agar surface prior to challenge inoculation with a 7-day old fungal mycelial plug. Mycelial growth (mm) from the plug was recorded daily for 3 days. The second technique was a slight modification of the first in that the bacterial culture suspension was spread directly over the entire agar surface in glass Petri dishes. Each plate was inverted over its respective lid which contained 30 ml chloroform and plates were sealed in ziplock freezer bags. Cell lysis occurred for 50 min with exposure to chloroform. Spent cells were scraped from the agar surface with a spatula using aseptic technique prior to challenge inoculation with a fungal plug as before.

Soils. Agricultural soils were obtained from fields in eastern and southwestern Virginia. The first soil was a Ross loamy sand (61% sand, 30% silt, and 9% clay), pH 8.0, with a low concentration of available iron, and contained the following

(mg/kg) (A & L Eastern Agricultural Laboratories, Inc., Richmond, VA): P, 4; K, 64; Mg, 64; Ca, 2100; and Mehlich 1 extractable Fe, 1. The second soil was a Suffolk sandy loam (77% sand, 14% silt, and 9% clay), pH 5.7, with a high concentration of available iron, and contained (mg/kg): P, 98; K, 100; Mg, 13; Ca, 180; and Mehlich 1 extractable Fe, 25. The Ross and Suffolk soils were amended with 30% and 50% (v/v) acid-washed beach sand, respectively, to improve drainage. Soils were spread in shallow plastic greenhouse flats and exposed to a steam/air mixture for 2 h to eliminate indigenous soilborne pathogenic organisms. Flats were stored on greenhouse benches for two wk to allow recolonization by saprophytic microflora.

Population dynamics/distribution patterns of wild-type and antibiotic-negative (*ant*) and siderophore-negative (*sid*) derivatives. A single *Pseudomonas* isolate was chosen for a detailed greenhouse study to compare distribution patterns of the wild-type strain, and (*sid*) and (*ant*) mutants in the absence of soil irrigation. Based on the results of the preliminary root colonization assay, *in vitro* antifungal agar-based bioassays, and ease of genetic manipulation (personal communication, Dr. George H. Lacy, VPI & SU), strain L-850 was chosen for further colonization studies. L-850 produces an orange water soluble pigment that absorbs ultraviolet light at 366 nm on mRSM without or with iron at concentrations ranging from 1 to 200 μM Fe^{+3}. Fatty acid profile analysis (MIDI, Newark, DE) identified L-850 as *Pseudomonas chlororaphis* (similarity index = 0.828; formerly *P. aureofaciens*). Bacterial suspensions were prepared as for the

preliminary bioassay except that cells were harvested at a turbidity of 0.74 at A_{640nm} (10^9 viable cells/ml) and cultures were resuspended in 0.1 M phosphate buffer (pH 7.0). Ross and Suffolk soils were placed in Conetainers® to within 3 cm of the rim. The Conetainers® were perforated with a nail to create 4 mm diameter holes uniformly over the entire surface to allow moisture equilibration. Conetainers® were submerged in water and allowed to drain, as described previously, prior to the introduction of two surface disinfested cotton seeds. One ml of bacterial suspension was pipetted over each seed to simulate an in-furrow application, and seeds were covered to a depth of 1.8 cm with the same soil. Control seed treatments consisted of plus or minus application of 1 ml phosphate buffer. Conetainers® were embedded to rim level in moist acid-washed beach sand within 21.5 by 17.5 cm (height by width) plastic buckets, four per bucket.

Four buckets, two per soil type, were used as moisture sensors by performing daily weight measurements to monitor evaporative moisture loss from the system. Moisture levels in soils and sand were maintained at just below field capacity by slow drip irrigation of the sand surrounding the Conetainers® when necessary. The soil surface was also misted every other day to prevent excessive evaporative water loss. Greenhouse temperatures averaged 26.9/21.1 C day/night. Supplemental lighting was supplied by high pressure sodium lamps (500 $\mu E\ m^{-2}\ s^{-1}$ PAR) to provide a 14/10 hour light/dark cycle. Buckets were rotated on the benches daily to ensure uniform environmental conditions for all treatments. Following emergence, cotton plants were thinned to one plant/Conetainer®. Three

plants were sampled from each treatment at 22, 36, and 50 DAP. Fifty days was the maximum growth period for cotton in Conetainers® to prevent space from becoming a limiting factor to root growth. Conetainers® and plants were treated as described previously to obtain root systems. After gentle shaking to remove all but the tightly adhering rhizosphere soil, the root system of each plant was dissected with a sterile scalpel into upper and lower tap and upper and lower laterals. Root sections were placed in vials containing 0.1 M phosphate buffer and shaken on a wrist action shaker at maximum setting for 20 min. Root sections were retrieved, placed between moist paper towels, and stored at 8 C for subsequent root area determinations. Population densities were determined by plating 100 µl aliquots from serial dilutions of root-rhizosphere cell suspensions on KB_K or KB_{KN}. Colonies were enumerated after 24 to 48 h incubation. Additional samples were plated on non-antibiotic amended KB to enumerate indigenous microflora. Root area (cm^2) determinations were obtained using the TIFF Image Analyzer (TIA) computer software program developed for our laboratory (Chapter III). A brief description of the program has been published previously (36). Bacterial colonization was expressed as cfu/cm^2 root surface area.

Experimental design and statistical analysis. Treatments were arranged as a completely randomized design on greenhouse benches, four Conetainers®/bucket (replication), three replications per treatment. Due to lognormal distributions of rhizosphere bacterial populations on the root surface, a log transformation was applied to all population values prior to analysis. A multivariate analysis of

variance (MANOVA) procedure was used to calculate an exact F statistic for the hypothesis of no significance for main effects and interactions employing the Wilks' Lambda procedure (Dr. Quentin Nottingham, Statistics Department, VPI & SU). Treatment means were separated by Duncan's Multiple Range Test (DMRT) when the F test was significant (P = 0.05).

Results

Selection and characterization of rifampicin (R) - nalidixic acid (N) - double antibiotic resistant pseudomonad isolates. Twenty biocontrol strains were screened for spontaneous double antibiotic-resistant mutants of each. Nineteen of the twenty wild-type pseudomonad isolates formed a lawn of colonies on KB amended with 20-60 and 20-140 µg/ml N or R, respectively (intrinsic resistance levels), after 24 h incubation. Extensive wild-type colony formation was suppressed at 80 and 160 µg/ml N and R, respectively. Double antibiotic-resistant mutants were obtained by selecting for spontaneous mutants resistant to one antibiotic followed by screening of the mutant population for resistance to the second antibiotic. One to two putative resistant colonies (per 20 plates) occurred for 19 of 20 isolates after 24 h incubation on KB_{N80} or KB_{R160}. Double mutants were restreaked on KB_{NR} to confirm mutational stability. In the first round of screenings, no colonies of L-854 formed on either KB_N or KB_R agar plates. After 7 days of repeated screenings with fresh aliquots from -80 C stocks, a single colony was recovered on R and N plates at concentrations of 80 µg/ml each.

Generation times for mutant and respective parental isolates were similar. Intermittent streakings on KB without antibiotics for one week under laboratory conditions demonstrated a lack of reversion to wild-type antibiotic sensitivity. All strains retained wild-type phenotypic characteristics described in Table 1. Stock cultures were prepared in 15% glycerol and placed at -80 C.

Preliminary greenhouse cotton root colonization bioassay. The twenty antibiotic-resistant strains were screened for the ability to migrate down the cotton root system in the absence of soil irrigation. Root systems (approximately 7.5-8 cm in length) and the seed coat if present were gently removed from the Conetainer® medium. Agar plates amended with the appropriate concentrations of R and N were subdivided into sections numbered 1 to 6 corresponding to the seed coat and 1.5 cm incremental sections (1 to 6) of the hypocotyl/root system. The 1.5 cm root sections or seed coat were imprinted in their respective numbered agar section. Agar section 1 contained the seed coat if available, section 2, the 1.5 cm hypocotyl, sections 3 to 5, 1.5 cm root sections distal to the hypocotyl, and section 6, the root apex. The majority of the strains were able to migrate a distance of 4-5 cm down the roots distal to the application point 14 DAP (agar section 4, Table 2). Agar sections 5 and 6 contained the segments closest to the root tip, with 6 including the 1.5 cm root apex. Eight of 20 strains developed colonies in section 5, and one strain, AD4-34, was able to migrate with the elongating root apex. All isolates were still associated with the seed coat at high population densities after 14 days. A uniform population density and distribution pattern was evident for

Table 2. Root-segment agar imprint bioassay for cotton root colonization efficacy of Rifampicin/Nalidixic acid double antibiotic-resistant pseudomonad biocontrol strains in the absence of soil irrigation

	Plate section number[1]					
Strain	1	2	3	4	5	6
L-849	++++[2]	+++	++	-	-	-
L-850	+++	+++	+++	+++	-	-
L-851	+++	+	+	+	-	-
L-852	+++	+++	+++	+++	-	-
L-853	++	+	+	+	-	-
L-854	++	++	++	+	+	-
L-855	+++	++	++	+	-	-
L-856	ND[3]	ND	ND	ND	ND	ND
L-886	+++	++	+++	++	+	-
L-890	++++	+++	+++	+++	-	-
L-892	+++	+++	++	-	-	-
G226	+++	+	+	+	+	-
M-17	++++	++++	+++	++	+	-
AD4-34	+++[4]	+++	+++	+++	++	++
TR-21	+++	+++	+++	++	+	-
Ral-3	+++	++	+	-	-	-
WSB15134	++++	+++	+++	+++	+	-
31-12	+++	+++	++	++	+	-
AC4-52	+++	+++	++	++	-	-

[1]Section 1=seed coat population (application site), 14 DAP (harvest). Sections 2-6 are 1.5 cm root segments distal from the seed coat, with 2=hypocotyl and 6=root apex.
[2](+)=small amount of colony growth 36 h after agar imprint of root segment; (++)=moderate but scattered; (+++)=uniform colonization within entire imprint trough; (++++)= profuse growth within entire imprint trough.
[3] ND= No data. Seeds rotted by bacterium.
[4] Some seedlings with stem and leaf distortion (growth regulator symptoms).

strains L-850, L-852, L-890, AD4-34 and WSB15134 (4-5 cm distance from seed coat; agar sections 2-4). Following treatment with strain L-856, all but one cotton seedling exhibited postemergence seedling rot. Treatment with AD4-34 delayed seedling emergence while germinated seedlings exhibited stem twisting and produced small thin cotyledons, symptoms indicative of growth regulator activity. Seed coat and root sections from CMC and water control treatments developed fluorescent and nonfluorescent colonies when plated on KB but not KB_{NR} indicating a lack of indigenous antibiotic-resistant populations.

Isolation and characterization of strain L-850 antibiotic-deficient mutants (*ant*).

Transposon mutagenesis of L-850, initiated to generate antibiotic-deficient mutants, produced kanamycin-resistant transconjugants at a frequency of 1.5×10^6 per initial recipient from four separate matings. Fewer than 0.3% of 5000 transconjugants were auxotrophic mutants. Loss of antifungal activity concomitant with the loss of orange pigment production and UV_{366} absorption, characteristic of phenazines (phz), occurred at a frequency of less than 0.2%. As *P. ultimum* was a more sensitive indicator of antifungal activity (fungicidal and fungistatic modes of action for *P. ultimum* and *R. solani*, respectively, data not shown), it was used in the initial screening (10 colonies/plate) of the 5,000 transconjugants. Following this screening, seven (*ant*) mutants were selected for the two agar diffusion bioassays. Growth of *P. ultimum* on bacteria-treated plates after 24 h incubation was 100 and 91% of the untreated control for two mutant strains, M-54 and M-

3002, respectively, indicating loss of antifungal activity. Loss of orange phenazine pigment production was correlated with loss of antifungal activity *in vitro*. Agar diffusion bioassays substituting KB for mRSM-Fe resulted in yellow-green fluorescent pigment production under ultraviolet light at λ_{254nm}. *P. ultimum* fungal growth was 88% inhibited (compared to nontreated control) by both mutants on KB plates, indicating retention of the wild-type phenotype for siderophore biosynthesis. Generation times were 0.94, 1.05 and 1.02 h for L-850, M-54, and M-3002, respectively. Restriction enzyme digestion of the 7.18 kb pUT plasmid with *Eco*RI produced the predicted two restriction fragments, 5.13 kb and the intact 2.05 kb mini-Tn*5* mobile element. Southern analysis of M-54 and M-3002 genomic DNA digested with *Eco*RI, and probed with the 2.05 kb *Eco*RI ^{32}P-labeled fragment, identified a single transposition site in each. Digestion of M-54 and M-3002 genomic DNA with *Sal*I or *Bgl*II followed by hybridization with the ^{32}P-labelled probe identified a single insertion site in the genome of each mutant.

Comparison of wild-type L-850, (*ant*) and (*sid*) mutant population dynamics/distribution patterns on greenhouse-grown cotton. An (*ant*) and (*sid*) mutant of L-850 were generated by Tn*5* mutagenesis to compare wild-type and mutant root colonization patterns in two soils over time. Bacterial populations (cfu/cm^2) in all cases were the combined values of both the rhizosphere and root surface as no attempt was made to differentiate between the two in this study. A comparison of overall mean bacterial population densities irrespective of soil, time interval or root section are presented in Table 3, for wild-type L-850, M-3002 (*ant*)

Table 3. Overall mean population densities pooled with respect to soil type, root section, and sampling interval (22, 36, 50 DAP) for the wildtype L-850NR, L-923 (sid^-), and M-3002 (ant^-) strains

Strain	Mean log (cfu/cm^2)
L-923	5.5 ± 0.7 a[1]
L-850NR	5.0 ± 0.9 b
M-3002	5.0 ± 0.8 b

[1]Values are ± S.D. Means with same letters do not differ significantly according to Duncan's Multiple Range Test (DMRT; P = 0.05).

Wilks' Lambda test statistic for the hypothesis of no overall effect of bacterial strain indicated a significant treatment difference (F = 16.09; P>F = 0.0001).

and L-923, a Tn*5*-mediated (*sid*) derivative of L-850 generated by Dr. George H. Lacy, VPI & SU. The overall cfu/cm^2 root area was significantly higher (P = 0.05) for the L-923 (*sid*) strain than either the wild-type or the (*ant*) mutant. Wilks' Lambda F statistic for main and interactive effects indicated significance for bacterial treatment (F = 16.09, Probability (P) > F = 0.0001), soil (F = 41.62, P > F = 0.0001), root section (F = 72.44, P > F = 0.0001) time (F = 12.68, P > F = 0.0001) and the interactions, treatment x root (F = 3.39, P > F = 0.003) and treatment x root x soil (F = 2.18, P > F = 0.03).

Bacterial population density and distribution patterns on each of four root sections in response to soil type are presented in Table 4. Bacterial populations in the Ross loamy sand (pH 8) were consistently higher on all root sections than in the pH 5.7 Suffolk sandy loam irrespective of sampling time. Differences in colonization efficiency between the two soils was greatest on upper lateral roots (3/4 log unit difference in population size). Lower tap roots in contrast, were least affected by differences in soil physical/chemical characteristics (1/8 log). As expected, bacterial migrational patterns displayed a gradient of declining density from upper tap to lower laterals, a trend that was statistically significant (P = 0.05). Bacterial strains in the Suffolk sandy loam tended to migrate in a more direct manner from the application site (seed) to the tap root and lower laterals as evidenced by higher population densities per unit root surface on the lower tap than upper laterals. In contrast, a more lateral distributional migration was

Table 4. Effect of soil type on bacterial population density/distribution patterns for each of four cotton root sections

	Log (cfu/cm^2)	
Root section	Ross loamy-sand	Suffolk sandy-loam
UT[1]	6.3 ± 0.3 a[2]	5.7 ± 0.6 a
UL	5.4 ± 0.4 b	4.7 ± 0.6 bc
LT	5.1 ± 0.7 c	5.0 ± 0.8 b
LL	4.7 ± 0.5 d	4.4 ± 0.6 c

[1]UT=Upper tap root, UL=Upper laterals, LT=Lower tap root, LL=Lower laterals.

[2]Values are the mean (n = 27) ± S.D. Means with same letters within a column do not differ significantly according to Duncan's Multiple Range Test (DMRT; P = 0.05).

demonstrated in the Ross loamy sand, as bacterial strains colonized upper laterals at significantly higher densities than on the lower tap.

Mean population sizes of individual strains did not fluctuate to any great extent (≤1/2 log unit) on cotton roots over the time interval sampled (22-50 DAP) (Fig. 1). Strain L-923 (*sid*) colonized root systems at higher densities than either wild-type or M-3002 (*ant*) strains at 22, 36, and 50 DAP (harvest). Populations migrated distal to the application site along the root surface as roots elongated. Density/cm^2 increased between 22 and 36 DAP on upper tap and upper lateral roots for bacterial treatments L-923 and M-3002, followed by a decline to density levels comparable to or just below initial sampling values by harvest, 50 DAP. The increase at day 36 indicated that population proliferation occurred rather than a spatial redistribution of the original population but only on these two root sections. Populations persisted after day 22 at levels between log 4 and log 6 cfu/cm^2, depending upon strain and root section, even as total root area increased 122% from day 22 to 50. Combining means of populations for both soils, L-923 (*sid*) proliferated to a greater extent on all root sections with the exception of the upper tap, once spatial migration was completed (Fig. 2), indicating less sensitivity to soil physical or chemical characteristics. Differences among strains were especially marked for the lower tap and lower lateral roots. Examining the effect of soil type on colonization efficiency, L-923 (*sid*) matched or exceeded population values of L-850 (wt) and M-3002 (*ant*) on all four root sections over time, with the exception of the upper tap densities in the Suffolk sandy loam [log 6.0 and log 5.7 cfu/cm^2

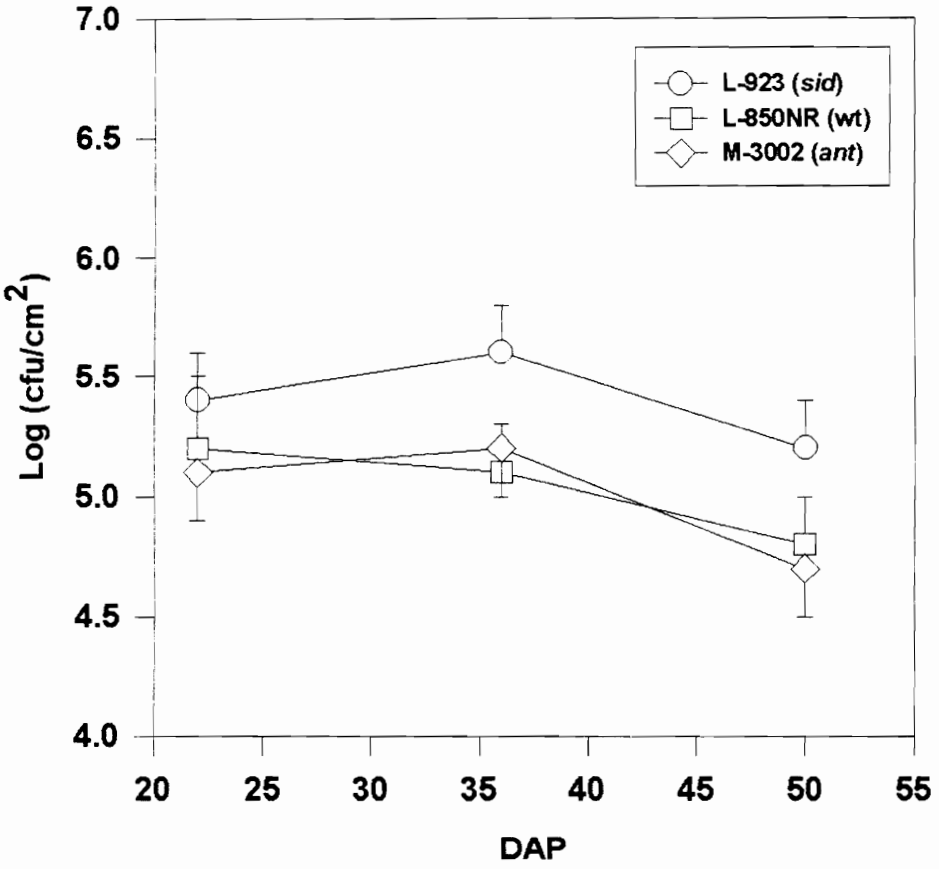

Figure 1. Cotton root bacterial populations [log_{10} (cfu/cm^2 root surface area)] determined at 22, 36, and 50 days after planting (DAP). Populations are the pooled overall mean values (plus or minus error bars) for both soils and all root sections.

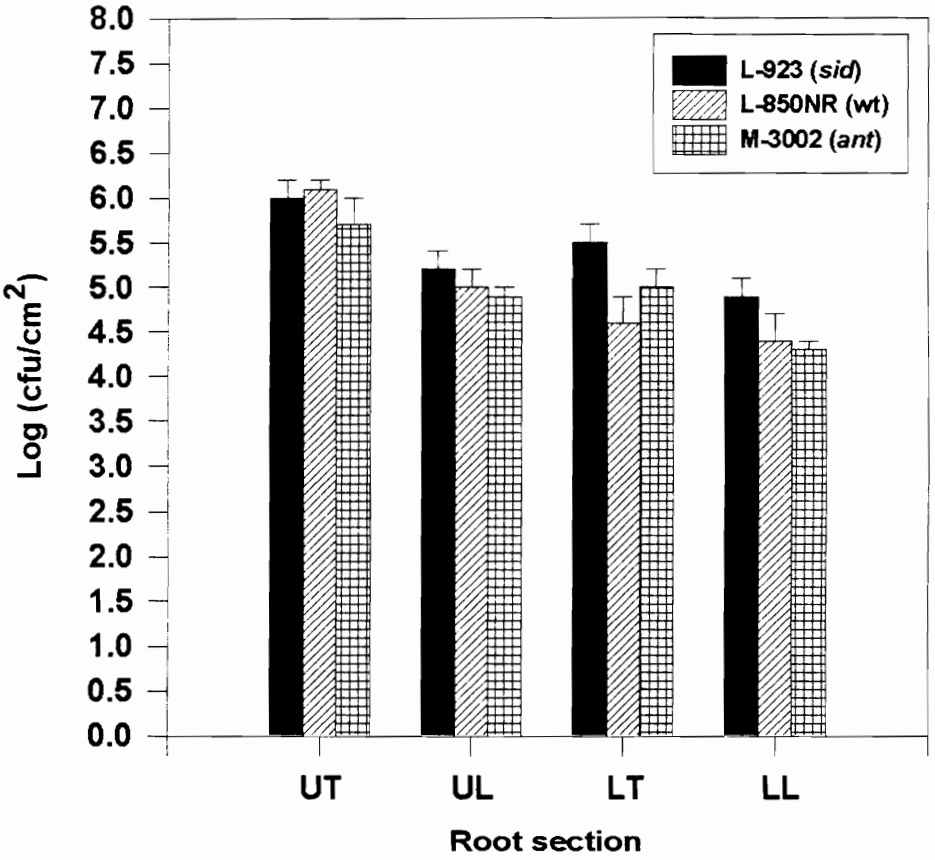

Figure 2. Comparison of bacterial population density/distribution patterns per unit surface area on four cotton root sections, upper tap (UT), upper laterals (UL), lower tap (LT) and lower laterals (LL). Values are the pooled means (+ S.E. bars) for both soil types and all three sampling intervals.

for L-850 and L-923, respectively (Fig. 3)]. Populations of all bacterial strains were initially detected on cotton roots at levels 1/2 to 1 log unit lower in the Suffolk sandy loamy at the first sampling period. Wild-type L-850 rhizosphere/root surface populations stabilized at densities in the range of log 5 to log 6 cfu/cm^2 on upper laterals and upper tap root sections, respectively, in the Ross loamy sand at all sampling intervals. In contrast, on lower tap and lower laterals, wild-type populations initially at log 5-6 cfu/cm^2, declined to log 3 cfu/cm^2 by harvest, a drop of greater than 150-fold. No consistent differences in migrational patterns or population densities on specific root sections could be established between L-850 (wt) and M-3002 (*ant*). Indigenous root microbe populations ranged from log 3.6 to log 4.5 cfu/cm^2 root surface for fluorescent and non-fluorescent aerobic bacteria, and from log 3.6 to log 4 fungal cfu/cm^2, the larger values present on lower lateral roots.

Comparisons of total plant root area (cm^2) in response to treatment for both soils combined at harvest are presented in Table 5. Although total root area values did not differ significantly among the bacterial strains, treatment of seed with bacteria promoted growth of cotton roots relative to the nontreated control ($P = 0.05$). The greater root area values with application of strain L-923 (*sid*) over that of the controls remained consistent between soil types. Growth promotion by L-923 (*sid*) was more pronounced in the Ross soil with percent increases of 48 and 19 respectively, for the Ross and Suffolk soils, over that of the nontreated control. Mean individual root section area values for all treatments combined at

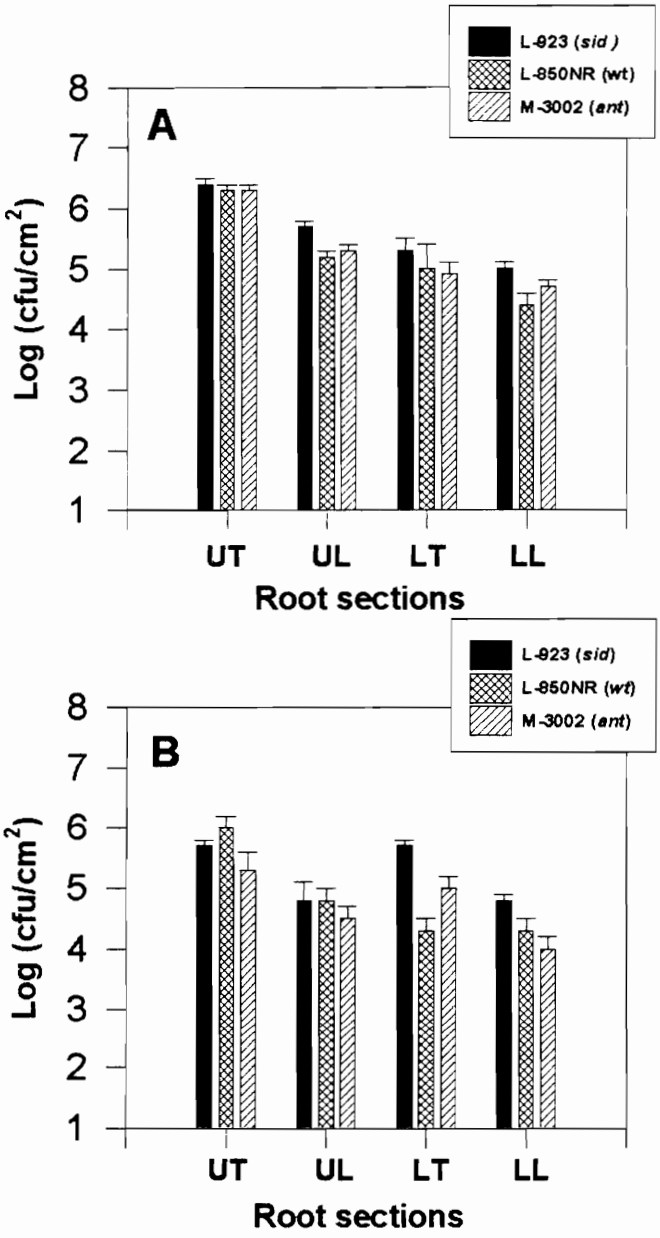

Figure 3. Comparison of cotton root bacterial population density/distribution patterns in the Ross loamy sand (A) and the Suffolk sandy loam (B) soils. Means are pooled values with respect to time.

Table 5. Comparison of total cotton root area (cm^2) at harvest (50 DAP) in response to bacterial seed treatment

Treatment	Total root area (cm^2)
L-923 (*sid*)	22.31 a[1]
L-850NR (wt)	19.07 ab
M-3002 (*ant*)	18.86 ab
Control[2]	16.49 c

[1]Mean pooled area values (n = 6) for both soils. Means with same letters not significantly different according to Duncan's Multiple Range Test (DMRT; P = 0.05).

[2]Uninoculated buffer control treatment.

harvest were 10.28, 7.05, 0.99, and 0.85 cm^2 for lower laterals, upper laterals, upper tap, and lower tap, respectively. Total root area for the nontreated controls did not differ between the two soils with mean values of 15.07 and 17.92 cm^2 for the Suffolk sandy loam and Ross loamy sand, respectively, indicating no significant effect of soil physical/chemical characteristics on cotton root growth.

Discussion

Rhizosphere populations of wild-type *P. chlororaphis* strain L-850 and mutant strains displayed similar migrational patterns on cotton roots, but densities varied significantly, dependent upon soil and position on the cotton root system. Mean population values across all strains and root sections indicated 3-fold higher cfu/cm^2 in the Ross loamy sand than the Suffolk sandy loam, reflecting the differences in soil chemical and physical properties.

Rhizosphere competence, defined by Ahmad and Baker (1) and modified by Weller (45), is the relative root-colonizing ability of a bacterial strain. The term root colonization in the present study included both the root surface and rhizosphere environments since separate densities for each were not determined. The original definition, however, did not include time as a critical component. Rhizosphere competence should therefore encompass not only growth, but survival and persistence of stable populations over some defined period of time. The results of the present study demonstrate various degrees of rhizosphere competence among L-850 (wt) and the mutant strains L-923 (*sid*) and M-3002 (*ant*) by their vertical

downward migration along cotton roots in the absence of soil irrigation. In addition, population proliferation occurred between 22 and 36 DAP on select root sections (upper tap, upper laterals) once migration ceased, followed by maintenance of relatively stable populations up to 50 DAP (harvest). Numerous studies have provided experimental evidence establishing soil irrigation as a dominant mechanism influencing passive bacterial movement (2, 13, 28, 37). At a fixed soil matric potential of -0.75 mPa, *P. fluorescens* 2-79RN$_{10}$ moved only 0.5 cm away from the point of application (38). Addition of irrigation water caused distribution of bacteria in more continuous longitudinal colonies along the root. *Pseudomonas chlororaphis* demonstrated rhizosphere competence in the absence of soil irrigation and would certainly yield a more uniform distribution with the application of irrigation water. Fluctuations in rainfall throughout the growing season would be less critical for root colonization provided there was sufficient soil moisture for bacterial migration. Plant growth promotion by *Pseudomonas* plant growth-promoting rhizobacteria (PGPR) was dependent on the colonization of lower lateral roots with high population levels, both achieved only by daily watering (13). Their studies concluded that soil moisture alone was insufficient to promote both colonization and growth promotion. Treatment of cotton seed with *P. chlororaphis* (mutants and wt) resulted in both colonization of lower lateral roots and root growth promotion as evidenced by a greater total root area (cm^2) relative to the uninoculated controls. Other than soil water, factors not investigated in the present study that influence passive movement of bacteria along a root surface include the

presence of capsular material (7), soil pore size distribution (32), continuity of water-filled pores in a specific size range (19), and diurnal shrinking and swelling of roots which creates gaps for more rapid and efficient dispersal in moist soil or water films on the root surface (17).

Rhizosphere bacterial enumerations on agar from serial dilutions of root sections can be misleading and may, at best, estimate only a fraction of the populations present in the rhizosphere and/or root surface. Soil moisture has been found to influence colony enumerations utilizing this technique, as wetter conditions produce a greater amount of adhering rhizosphere soil, resulting in overestimations of rhizosphere bacterial numbers (44). In contrast, underestimation of root populations can occur when many small fine roots are left behind during lifting and processing of root systems from bulk soil (2). The latter situation was a concern in the present study for root systems extracted from the Ross loamy sand, a factor that may have produced smaller estimates of rhizosphere or root surface populations.

Populations of L-923 (*sid*) were greater than those of L-850 (wt) or M-3002 (*ant*) on all root sections, a trend that appeared independent of soil physical/chemical characteristics. Once populations of L-923 (*sid*) reached a stable maintenance density following downward migration at 22 DAP, less fluctuation occurred, notably on lower tap and lower lateral roots which contained the highest populations of competitive resident microbes. Moreover, mean root population density values on all root sections were higher for L-923 (*sid*) in both

soils after 50 days (harvest) than either the wild-type or (*ant*) mutant, reflecting perhaps an increased survival capacity as well as increased colonization efficiency. The greater rhizosphere competency exhibited by this mutant may be attributed to the absence of siderophore biosynthesis. The Tn*5* insertion site in the genome of L-923 (*sid*) may have blocked one of the initial steps in the biosynthetic pathway, resulting in a significant decrease in metabolic energy expenditure required for siderophore production. Strain L-923 (*sid*) still exhibited antifungal activity *in vitro* against *P. ultimum* and *R. solani* and was presumably unaltered in the production of antibiotics synthesized by L-850 (wt) including one or more phenazines, although this was not verified at the genetic level. Loper (30) also obtained (*sid*) mutants of a *P. fluorescens* strain with a cotton spermosphere colonization-enhanced phenotype over that of the wild-type. Likewise, two of five *P. fluorescens* mutants deficient in production of an antifungal compound (*afu*) colonized cotton roots at significantly higher (P = 0.05) populations than the wild-type (22). However, both studies assessed colonization for only two and 12 DAP, respectively.

The majority of studies investigating colonization of mutants deficient in the production of one or more secondary metabolites have concluded that mutant strains have retained colonization capabilities equal to that of the wild-type (10, 18, 22, 23, 28, 30, 42). All of these studies, however, monitored colonization for short time intervals ranging from two to 21 DAP. Results from these studies have led researchers to question the role of secondary metabolites in the natural ecology

of the producing organism (34). In the current study, differences in rhizosphere competence between L-923 (*sid*) and L-850 (wt) on cotton became more apparent with sampling times after day 22, emphasizing the need for more long-term colonization data. Only a very limited number of studies have focused on differences between mutant and wild-type strains throughout an entire growing season (3) or several successive growing seasons (34). Bakker et al (3), monitored populations of *P. putida* wild-type and (*sid*) mutant strains applied to potato seed pieces for 130 DAP. Results, however, supported those of short term observations. At least one study has proposed a role for secondary metabolite production in the enhancement of both colonization and long-term survival over three or five successive 20-day cycles of wheat growth (27). Populations of phz^- strains declined more rapidly than phz^+ strains of *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 on wheat roots due to competition from indigenous microbes after 70 days, a decline not evident in short-term studies. Thus phenazine antibiotic production appeared to contribute to survival of the producing strain in the rhizosphere environment. Data with L-923 (phz^+) appears to support a role for phenazine biosynthesis in root colonization.

Soil physical and/or chemical properties influenced bacterial densities of all three strains (wild-type, *sid* and *ant*) in the present study but did not significantly affect distribution along the cotton root surface, *i.e.*, populations were able to colonize cotton roots from the upper tap to the lower laterals, but densities differed among root sections in the two soil types. Soil pH alone may have influenced

population levels of the three strains directly, assuming that the rhizosphere pH more or less reflected that of the bulk soil due to the absence of fertilizer treatment. Low soil pH has been directly correlated with a reduction in survival and nodulation by strains of *Rhizobium* (16). Interestingly, colonization of wheat roots by *P. fluorescens* 2-79 was greater at a rhizosphere pH of 6 to 6.5 than at more alkaline soil pH's, although growth *in vitro* was optimum at neutral pH or above, an observation attributed to less competition from resident microbes at the lower pH's (45). Alternatively, soil pH may indirectly influence bacterial rhizosphere densities via a pH-induced alteration of exudate materials released from the plant or by changes in ionic solubility in the soil solution, thus, increasing/decreasing availability of essential elements (4, 20). Following a reduction in soil solution pH, iron deficient sunflower plants exuded increased amounts of riboflavin and other uncharacterized compounds that reduced Fe^{+3} to Fe^{+2} (4).

Results of studies with peas grown at pH 5.8 (29) and wheat grown in soil as low as 5.3 (5), support our observations of bacterial migration and colonization of roots even in low pH soil. Detrimental effects of an acid soil pH on growth of cotton roots directly appear doubtful in view of the fact that total cotton root area 50 DAP for the nontreated controls did not differ significantly between soils, and that cotton grows worldwide over a broad range of soil pH.

The significant root growth promotion by L-923 (*sid*) was consistent throughout this study and in preliminary colonization bioassays even in the absence of root pathogens. Increases of 14 to 48% were observed for treatment with bacteria in

general, but the smallest root increase was induced by treatment with mutant M-3002 (*ant*). Growth promotion, both in the presence and absence of root pathogens has been reviewed extensively (25, 26). PGPR produce metabolites which may stimulate root growth directly (25), or indirectly though the inhibition of indigenous nonparasitic bacteria and fungi that produce mild phytotoxins harmful to plant growth (12). Production of phenazine antibiotic compounds has been documented to exert a toxic effect on indigenous soil bacteria, pathogenic fungi, algae and higher plants (43). The majority of studies documenting a role for phenazine biosynthesis in wheat root colonization and take-all pathogen suppression have been conducted under acidic soil conditions (10, 34, 42). Data by Brisbane et al. (8) indicated that phenazine-1-carboxylic acid has no inhibitory activity in environments above pH 7.0. Thus enhanced colonization through competitive exclusion of deleterious indigenous microorganisms should be greatly enhanced on roots growing in acid soil. This observation was supported in the present study by the consistently lower population densities for the phenazine-deficient mutant M-3002 on lower lateral roots at all sampling intervals in the pH 5.7 Suffolk soil where populations of and competition from indigenous microbes was greatest. In addition, the population density mean pooled with respect to root section was 1.2 log units lower in the Suffolk soil relative to that in the Ross soil at the first sampling interval (Appendix Table 12), emphasizing perhaps a role for phz biosynthesis in the early stages of host root colonization. The total root system population of M-3002 (*ant*) at harvest was lowest among the three strains

in the Suffolk soil possibly indicating a reduced survival capacity as well. Decreased colonization efficiency of M-3002 (*ant*) was also reflected in a decreased suppression of the cotton damping-off pathogens *P. ultimum* and *R. solani* (Chapter V).

The absence of siderophore biosynthesis appeared to enhance the rhizosphere competence of *P. chlororaphis* in the present study. In addition, the ability to maintain a more uniform root distribution pattern of higher population densities than the wild-type strain may have contributed to the significant root growth promotion by L-923 (*sid*). Rather than genetically modifying a bacterium for synthesis of numerous secondary metabolites to develop a "superior biocontrol strain", research should focus on characterization of host plant root exudates and their effect on the dynamics of colonization and antibiotic production by introduced biocontrol isolates in the rhizosphere. Such detailed information obtained for each individual biocontrol strain/soilborne pathogen/plant host system will allow "streamlining" or elimination of mechanisms deemed unimportant or ineffective for successful biological control within that particular system, as well as improve the chances of selecting strains with maximal rhizosphere competence.

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V.

ANTIBIOTICS AND SIDEROPHORES: ROLE IN THE BIOLOGICAL CONTROL OF COTTON SEEDLING DISEASE BY *PSEUDOMONAS CHLORORAPHIS*

Abstract

Pseudomonas chlororaphis strain L-850 produces siderophores (sid^+) and several fungicidal antibiotics (ant^+) with activity against *Pythium ultimum* Trow and *Rhizoctonia solani* Kühn, causal agents of cotton (*Gossypium hirsutum* L.) seedling disease. Soil inoculum levels of *P.ultimum* and *R.solani* were manipulated to establish three levels of pathogen pressure, low (PIII), intermediate (PII), and high (PI) in two soils (pH 5.7, high iron, high phosphorus; pH 8.0, low iron, low phosphorus) producing cotton seedling stands in a greenhouse study ranging from 2 to 99% emergence. Three transposon-mediated derivatives of strain L-850, a single (*sid*) and two (*ant*) mutants, were generated to assess the contribution of siderophores and antibiotics to pathogen suppression, and to determine limitations on the effectiveness of *P. chlororaphis* in controlling both pre- and postemergence damping-off (disease phase 1) and hypocotyl/root rot symptoms produced on more mature plants (disease phase 2). Percent seedling emergence, hypocotyl/tap root lesion severity (DSHT), root rot severity (DSRR), and foliar fresh weight differed in response to bacterial seed treatment (mutants vs wild-type). Soil pH did not appear to greatly influence pathogen suppression although phase 2 disease

severity ratings were slightly higher in the pH 5.7 soil regardless of bacterial treatment. Phase 1 pathogen suppression by *P. chlororaphis* L-850NR, a Nal-Rif spontaneous resistant mutant of L-850, equalled (P = 0.05) that provided by the recommended fungicide seed treatment at intermediate (PII) and low (PIII) soil pathogen pressure, but not under high pressure (PI). Phase 2 suppression of DSRR by *P. chlororaphis* L-850 was comparable (P = 0.05) to the fungicide only at low (PIII) soil inoculum density, whereas suppression of DSHT by L-850 was equal to the fungicide at both intermediate (PII) and low (PIII) inoculum levels. For PI, PII, and PIII, control of both disease phases 1 and 2 was comparable (P = 0.05) between the wild-type L-850 and (*sid*) mutant, L-923, whereas a reduction in phase 2 suppression was exhibited by the (*ant*) mutant M-3002. These results support a role for antibiotic but not for siderophore biosynthesis in the biological control of *P. ultimum* and *R. solani* on cotton by *P. chlororaphis*.

Introduction

Cotton seedling disease complex caused by *Pythium ultimum* and *Rhizoctonia solani* occurs worldwide in soils ranging in pH from 4.5 to 8.5 (18). There are two important phases of this disease, the pre- and postemergence damping-off that results in premature seedling mortality (phase 1), and the hypocotyl/root rot and root lesion formation on surviving seedlings and more mature plants (phase 2) that may result in substantial reductions in cotton root area and subsequent losses in yield (4). A prerequisite of successful bacterial biocontrol of both phases is

extensive colonization of cotton roots with adequate population densities (rhizosphere competency) to provide both short-term and extended pathogen suppression. Concomitant with rhizosphere competence is the biosynthesis of antifungal secondary metabolites over the pH range in which the pathogens operate.

Secondary metabolites synthesized by fluorescent pseudomonads with specific activity against *P. ultimum* and *R. solani* come under the broad classification of antibiotics (5, 7, 8, 9, 10, 11, 12, 17) and siderophores (2, 16). To ascertain the relative importance of siderophores and antibiotics in the control of cotton seedling pathogens, pseudomonads that produce both groups of compounds were provided though a cooperative agreement between Ecogen Inc. and VPI & SU. Antifungal *in vitro* agar bioassays with *Pseudomonas chlororaphis* strain L-850, resulted in 80-100% inhibition of mycelial growth of *P. ultimum* and *R. solani* at iron (Fe^{+3}) and phosphorus (P) concentrations ranging from 0-200 µM and 0-100 mM, respectively. Strain L-850 produces siderophores and multiple uncharacterized antibiotics, including one or more phenazines. Greenhouse colonization studies to date have demonstrated differences in rhizosphere competence on cotton among wild-type L-850, and antibiotic-deficient (*ant*) and siderophore-deficient (*sid*) mutants of L-850, in a Suffolk sandy loam (pH 5.7; high Fe^{+3}, high P) and a Ross loamy sand (pH 8.0; low Fe^{+3}, low P) soil (20, Chapter IV). The elimination of siderophore biosynthesis appeared to enhance the root colonizing ability of *P. chlororaphis*. Previous studies on cotton have evaluated siderophores as a

suppressive mechanism of *P. fluorescens* strain 3551 by comparing wild-type and (*sid*) mutants. There were demonstrated differences in cotton seedling emergence, but these differences were less evident in protection against cotton seed infection and colonization by *P. ultimum* (16). It appears that more than one mechanism (antibiotics, competition, etc.) is operating in suppression of *P. ultimum* on cotton. The goals of this study were to: i) compare L-850 (wt), L-923 (*sid*) and M-3002 and M-54 (*ant*) derivatives of L-850 for *P. ultimum* and *R. solani* suppression in an acidic soil (pH 5.7) with high concentrations of Fe^{+3} and P, and an alkaline soil (pH 8.0) with low concentrations of Fe^{+3} and P; ii) evaluate biological control efficacy at three soil pathogen inoculum densities; and iii) assess the ability of a rhizosphere competent strain (*P. chlororaphis)* to provide both short-term (phase 1) and extended (phase 2) biological control. A preliminary report has been published (21).

Materials and Methods

Bacterial strains and culture media. *Pseudomonas chlororaphis* L-923, M-3002, and M-54 are, respectively, a (*sid*) and two (*ant*) Tn*5*-mediated mutants of wild-type strain L-850. The generation of the mutants and their phenotypic characteristics have been described previously (Chapter IV). The wild-type strain produces secondary metabolites with *in vitro* activity against the cotton root rot pathogens *P. ultimum* and *R. solani*. Strain L-850NR is a spontaneous mutant of L-850 resistant to rifampicin and nalidixic acid, and is wild-type for growth,

siderophore, antibiotic and pathogen suppression. Growth media used included King's medium B (KB; 13) and modified RSM-Fe (mRSM-Fe). RSM is a rhizosphere medium developed by Buyer et al. (3) and was modified to reduce the amount of extracellular polysaccharides produced by some pseudomonads and to enhance *in vitro* antifungal activity. Revised components and concentrations included 1% glucose, 0.5% casamino acids, 1.56 µm $MnSO_4 \cdot H_2O$, and 100 µM Fe^{+3} supplied as diethylenetriamine pentaactic acid (DTPA). Antibiotics, when appropriate, were added to the media at the following concentrations (µg/ml): kanamycin, 50; nalidixic acid, 80; and rifampicin, 160.

Bacterial inoculum. Stock cultures of biocontrol strains stored at -80 C (15% glycerol) were streaked onto KB or mRSM-Fe and incubated at 28 C. A single colony from an overnight culture of each strain was suspended in 15 ml mRSM-Fe containing the appropriate antibiotics in a shaker-incubator (120 rpm) for 8 h at 28 C. A 1 ml aliquot of each was placed in 110 ml mRSM-Fe and incubated overnight as above. Cultures were pelleted by centrifugation, washed once with 0.1 M phosphate (P) buffer (pH 7.0) and resuspended in 110 ml buffer. Turbidity (Beckman model DU-64 Spectrophotometer) for each strain was adjusted to 0.7 at 640 nm which corresponded to 10^9 viable cells/ml.

Preparation of pathogen-infested soils. The first soil was a Ross loamy sand, pH 8.0, with a low concentration of available iron, and contained the following (mg/kg soil): P, 4; K, 64; Mg, 64; Ca, 2100; and Fe, 1. Texture analysis showed the Ross soil to consist of 61% sand, 30% silt, and 9% clay. The second soil was

a Suffolk sandy loam, pH 5.7, with a high concentration of available iron, and contained (mg/kg soil): P, 98; K, 100; Mg, 13; Ca, 180; and Fe, 25. The Suffolk sandy-loam was 77% sand, 14% silt, and 9% clay. The Ross and Suffolk soils were amended with 30% and 50% (v/v) acid-washed beach sand, respectively to improve drainage.

Twenty-five g of foxtail millet (*Setaria italica*) was rehydrated in flasks with distilled water (25 ml) for 3 h and autoclaved for 20 min, followed by a 60 min sterilization 24 h later. PDA plugs (4 mm) from 7-day old *P. ultimum* and *R. solani* cultures were placed in flasks, four plugs/pathogen and incubated at 25 C for 7 days. Flasks were shaken daily for 30 sec to ensure uniform fungal distribution and to prevent clumping. Fifty infested millet seeds (for each pathogen) were retrieved from the flasks and plated on PDA. After 24 h 100% of the millet seeds had produced colonies for both pathogens. Ross and Suffolk pathogen-infested stock soils were produced by mixing 25 and 12.5 mg of *P. ultimum* and *R. solani*-infested millet, respectively, per 1200 cc of steam sterilized soil. These quantities of inoculum produced 2 to 10% post-emergence seedling stands (severe pathogen pressure, PI) in preliminary dose-response trials (data not shown). Following infestation, soils were maintained at 22 C in a greenhouse for two wk prior to use. The PI pathogen density in each soil was diluted 1:14 and 1:400 with known volumes of steam sterilized soil and mixed thoroughly in a 19-L seed treater to obtain intermediate (PII) and low (PIII) pathogen densities respectively, in each soil. These soil pathogen levels generated cotton seedling

stands in the range of 2 to 99% for each soil type (Table 1). In-furrow cotton seed treatment applications for PI, PII and PIII were always initiated no later than 48 h following soil dilutions. Pathogen enumerations (propagules/g oven-dried soil) at planting were obtained using selective media whose constituents and concentrations were based on the diversity of saprophytic microflora encountered in soil dilution platings. *P. ultimum* populations were determined by counting numbers of hyphae emerging from 50 µl droplets obtained from a series of soil dilution suspensions onto 2% water agar (WA) supplemented with the following (mg/L): benomyl (Benlate® 50WP), 20; and pentachloronitrobenzene (Terrachlor® 75WP), 146. *R. solani* hyphae were enumerated from 100 µl droplets as for *P. ultimum* above on 2% WA containing (µl/L): prochloraz (Prochloraz 40EC), 21; and metalaxyl (Subdue® 2E), 31.

Greenhouse treatments. Acid-delinted cotton seeds, 'Deltapine 50' were surface disinfested (2 min, 1.7% sodium hypochlorite) and dried for 12 h in a laminar flow hood prior to planting. A pentachloronitrobenene (PCNB)/metalaxyl (Apron®/Terraclor® D 25/6%) fungicide treatment was applied at the recommended rate of 227 g/45.4 kg seed. Seeds were lightly coated with canola vegetable oil using a chromatography spray atomizer (5 p.s.i.), placed in a glass jar containing the fungicide, and shaken until uniformly coated. Plastic greenhouse trays 40 cm (l) x 30 cm (w) x 7.5 cm (h) were subdivided into four quadrants using polyurethane treated polystyrene R-3 insulation sheathing (Dow Chemical Co.; Midland, MI) and filled with soil to within 1 cm of the tray rim. To standardize

Table 1. *Pythium ultimum* and *Rhizoctonia solani* inoculum density levels (PI, PII, PIII) in the Ross loamy sand (pH 8.0) and the Suffolk sandy loam (pH 5.7) prior to planting

	Soil population densities(# p/g)[1]					
	Ross loamy-sand			Suffolk sandy-loam		
Pathogen	PI	PII	PIII	PI	PII	PIII
P. ultimum	355	330	27	25	0.9	0.8
R. solani	7	4	< 1	< 1	< 1	< 1

[1]Propagules/g oven-dried soil (determined by plating on selective medium) . PI soils were diluted 1:14 (PII) and 1:400 (PIII) to produce stand counts ranging from 2 to 99% (PI - PIII).

planting distance between seeds (3.8 cm; 18) in each quadrant, a cardboard template with holes 3.8 cm apart was placed on the soil surface. Cotton planting at this spacing resulted in a density of 24 seeds/quadrant with seeds deposited into holes 1.8 cm in depth. A one ml aliquot (10^9 cells) of bacterial suspension was pipetted over each seed before covering with infested or noninfested soil. Controls consisted of 1 ml P buffer applied to seeds in pathogen non-infested soil (nontreated control), or pathogen infested soil (nontreated pathogen control). Tests at pathogen densities PI, PII and PIII were initiated at staggered but overlapping time intervals for ease of data sampling. Temperatures averaged 26.9/21.1 C day/night. Soil moisture was maintained at or near field capacity, 17.6 and 12.4% moisture, for the Ross and Suffolk soil respectively, by daily watering. Supplemental lighting was supplied by high pressure sodium lamps (500 μE m^{-2} s^{-1} PAR) to provide a 14/10 hour light/dark cycle.

Data collection and statistical procedures. Disease incidence (% seedling emergence) was evaluated at 7, 14 and 21 days after planting (DAP). Emergence data for PI (high) and PII (intermediate) were transformed to their respective arcsine values prior to analysis because percentages covered a wide range of values (22). Plant growth data collected included root fresh weight (RFW), root area (RAREA, cm^2), foliar fresh weight (FFW), and foliar dry weight (FDW) for each plant at harvest, 42 DAP. Disease severity was evaluated on individual plants employing a rating scale of 1 to 6 for both % root rot and % hypocotyl/tap lesions with 1 representing 0, and 6, 81 to 100% of surface area with symptoms.

Foliar dry weights were recorded after drying plant material for 72 h at 70 C. Roots were wrapped in moist paper towels following fresh weight measurements and stored at 8 C until scanned for root area determination. Total root area (cm^2) for each plant was obtained using a TIFF Image Analyzer (TIA) software program (19). Values were used to calculate mean percent reductions in root area in response to soil pathogen density and for treatment comparisons.

Tests were analyzed as a split-plot design with soil and pathogen density as whole plot factors and seed treatments (6 replicates total/treatment) as the split-plot factor. The general linear models (GLM) procedure was used to test main effects of soil, pathogen and treatment as well as interactions among factors. Data were pooled in some cases (main effects not significant), prior to mean separation by Duncan's Multiple Range Test (DMRT) when the overall F test was significant (P = 0.05).

Results

Pre- and postemergence damping-off control (phase 1). Wild-type, siderophore- and antibiotic-deficient mutants of *P. chlororaphis* were evaluated for their ability to suppress phase 1 damping-off. Propagules/g soil of *P. ultimum* and *R. solani* were determined prior to planting and were found to be similar for the two soils (Table 1), as determined by enumerations on selective media. The ability of the various bacterial treatments to suppress the pre- and postemergence damping-off disease phase (phase 1) was dependent on pathogen inoculum density (Table 2). Percent emergence was evaluated at 14 (data not presented) and 21 DAP. Mean

Table 2. Effect of seed treatments on cotton seedling emergence (% of nontreated control) 21 DAP pooled relative to soil, at three soil inoculum levels of *Pythium ultimum* and *Rhizoctonia solani* (PI, PII, PIII)

	Percent Emergence		
Treatment	PI	PII	PIII
PCNB/metalaxyl	79.8 (93.7) a[1]	76.1(91.4) a	(102.6) a
L-850 wt	41.1(43.3) b	52.5 (62.5) b	(90.3) a
L-850 NR	37.0 (38.3) b	61.5 (71.4) ab	(93.8) a
L-923 (*sid*)	43.4 (47.1) b	57.1 (70.2) b	(74.0) a
M-54 (*ant*)	36.9 (36.8) b	47.9 (54.8) b	(102.5) a
M-3002 (*ant*)	41.4 (43.1) b	52.6 (62.0) b	(102.6) a
Pathogen alone	14.6 (8.9) c	43.7 (48.5) b	(99.9) a[2]

[1]Pooled emergence data for both soils. Percent emergence is presented as the arcsine transformation for pathogen levels PI and PII only. Emergence means within columns do not differ significantly (P = 0.05), Duncan's Multiple Range Test (DMRT). Untransformed data are in parentheses.

[2]Mean of nontreated pathogen control values for both soils (low pathogen pressure).

values presented are the pooled data for both soils due to a non-significant soil effect (P = 0.05) within each pathogen density level. At high (PI) and intermediate (PII) pathogen levels, all bacterial treatments increased seedling stands significantly (P=0.05) relative to the nontreated pathogen control, however, pathogen suppression was not equivalent to the standard fungicide seed treatment except for L-850NR at PII (intermediate). Treatment with the (*sid*) mutant L-923 produced the highest stand counts at PI (high) but the increase was not significantly greater than wild-type L-850 or (*ant*) mutant treatments, M-54 and M-3002. At the lowest pathogen density level (PIII), all treatments resulted in seedling stands equivalent to the fungicide.

Cooler greenhouse temperatures (15-22 C) for 96 h during the emergence phase of PIII delayed seedling germination by 5 days over that in PI and PII (7 vs 13 DAP). The delay in germination appeared to improve protection by the two (*ant*) mutants M-54 and M-3002 as evidenced by emergence values (over 100%) relative to the two controls (nontreated and nontreated pathogen, Table 2), although the increase was not statistically significant. In contrast, the delay in germination appeared to reduce the ability of the (*sid*) mutant L-923 to protect emerging seedlings (74% seedling stand), the protection provided in this case not equivalent to that provided at levels PI (high) and PII (intermediate).

Post harvest evaluation of plant growth variables. The effect of bacterial treatment on the plant growth variables, root fresh weight (RFW), root area (RAREA), foliar fresh weight (FFW), and foliar dry weight (FDW) was evaluated at

42 DAP (harvest). The response varied with respect to soil, pathogen pressure, and bacterial treatment (Table 3). Root fresh weight (RFW) was not influenced by soil (DMRT, P=0.05), indicating no direct effect of soil physical/chemical characteristics on cotton root growth. Cotton RFW was greater (P=0.05) at pathogen level PIII (low) but did not differ between PI (high) and PII (intermediate). Root fresh weight increased 55% under PIII (low) conditions relative to those in PI (high). There was no effect of bacterial treatment on RFW, although mean values for PI, PII, and PIII combined ranged from 0.6113 g for treatment with M-3002 to 0.752 g for plants treated with wild-type L-850 (data not shown).

Root area (RAREA; cm^2) exhibited results similar to RFW relative to soil and bacterial seed treatment (P=0.05). Root areas, as expected, were highest for cotton at low (PIII) pathogen density (pooled value of 25.28 cm^2; P=0.05, DMRT). Root area values for plants exposed to PIII (low) pathogen levels were 2-fold higher than plants at PI (high). Overall pooled mean root area values across all pathogen inoculum densities ranged from 17.63 cm^2 for the (*ant*) mutant M-3002 to 22.27 cm^2 for wild-type L-850 (data not shown). Treatment with L-923 (*sid*) provided a mean root area value similar to wild-type L-850 (21.46 cm^2). Although not significant, these values represented an 8 and 12% increase over the nontreated pathogen control for treatment with L-923 and L-850, respectively, and 7 and 11% increases respectively, over the nontreated control.

Table 3. Cotton plant growth variables, root fresh weight, root area, and foliar fresh and dry weight at harvest 42 DAP, in response to soil, inoculum densities of *P. ultimum* and *R. solani,* and seed treatment

Growth response variable	Soil[1]	Inoculum density[2]	Seed Treatment[3]
Root fresh weight (g)	NS[4]	** PIII 0.832 a PII 0.579 b PI 0.535 b	NS
Root area (cm^2)	NS	** PIII 25.28 a PII 17.81 b PI 12.42 c	NS
Fresh weight (g)	** 8.0 1.874 a 5.7 1.355 b	*** PIII 1.638 b PII 1.922 a PI 1.292 c	* L-850wt 1.785 a M-54 1.770 ab L-923 1.748 ab L-850NR 1.715 ab M-3002 1.677 ab NP 1.628 bc F 1.527 cd N 1.487 d
Dry weight (g)	** 8.0 0.429 a 5.7 0.291 b	*** PIII 0.410 a PII 0.389 a PI 0.269 b	NS

[1]Ross loamy-sand (pH 8.0) and Suffolk sandy-loam (pH 5.7). Main effect of soil on the four growth variables.
[2]Pathogen soil inoculum densities PI (high), PII (intermediate), and PIII (low) (see Table 1). Main effect of pathogen.
[3]L-850 = w.t. *P. chlororaphis*; L-850NR = rifampicin/nalidixic acid resistant mutant; L-923 = (*sid*) mutant; M-54 and M-3002 = (*ant*) mutants) F = PCNB/metalaxyl fungicide; NP = nontreated pathogen control; N = nontreated control. Mean values presented are pooled with respect to soil and pathogen.
[4]NS = Not significant.
*** (P = 0.01); ** (P = 0.05); * (P = 0.10). Means with same letters do not differ significantly, (DMRT) within each individual growth variable.

Foliar fresh weight (FFW) was the only growth response variable dependent on all three main effects (soil, pathogen density, seed treatment). Soil and pathogen inoculum density were significant (P=0.05) and highly significant (P=0.01), respectively. All bacterial treatments induced fresh weight increases of 12 to 20% over that of the nontreated control and from 3 to 9.6% over that of the nontreated pathogen control. Seed treatment differences were significant at P = 0.10. Treatment with *P. chlororaphis* mutant and wild-type strains increased FFW relative to the standard fungicide treatment as well.

Foliar dry weight (FDW) was dependent on soil and pathogen density (P=0.01), but was not influenced by seed treatment. Dry weight measurements exhibited a response to individual seed treatment similar to FFW but differences were significant only at the 12% level. Treatment with bacteria in general increased FDW compared to nontreated control plants with the exception of the (*ant*) mutant M-3002.

Post harvest evaluation of plant root rot/hypocotyl-tap lesion control (phase 2). *Pseudomonas chlororaphis* wild-type and mutant strains were evaluated for their ability to suppress phase 2 of the damping-off disease complex. Figure 1 provides a comparison of final cotton stands at harvest (42 DAP) for selected treatments in response to soil and pathogen pressure. Final stand counts reflected post-emergence percentages (21 DAP) for the majority of seed treatments indicating no further plant mortality after 21 days with the exception of the nontreated pathogen control. Stand differences between treatments were greatest

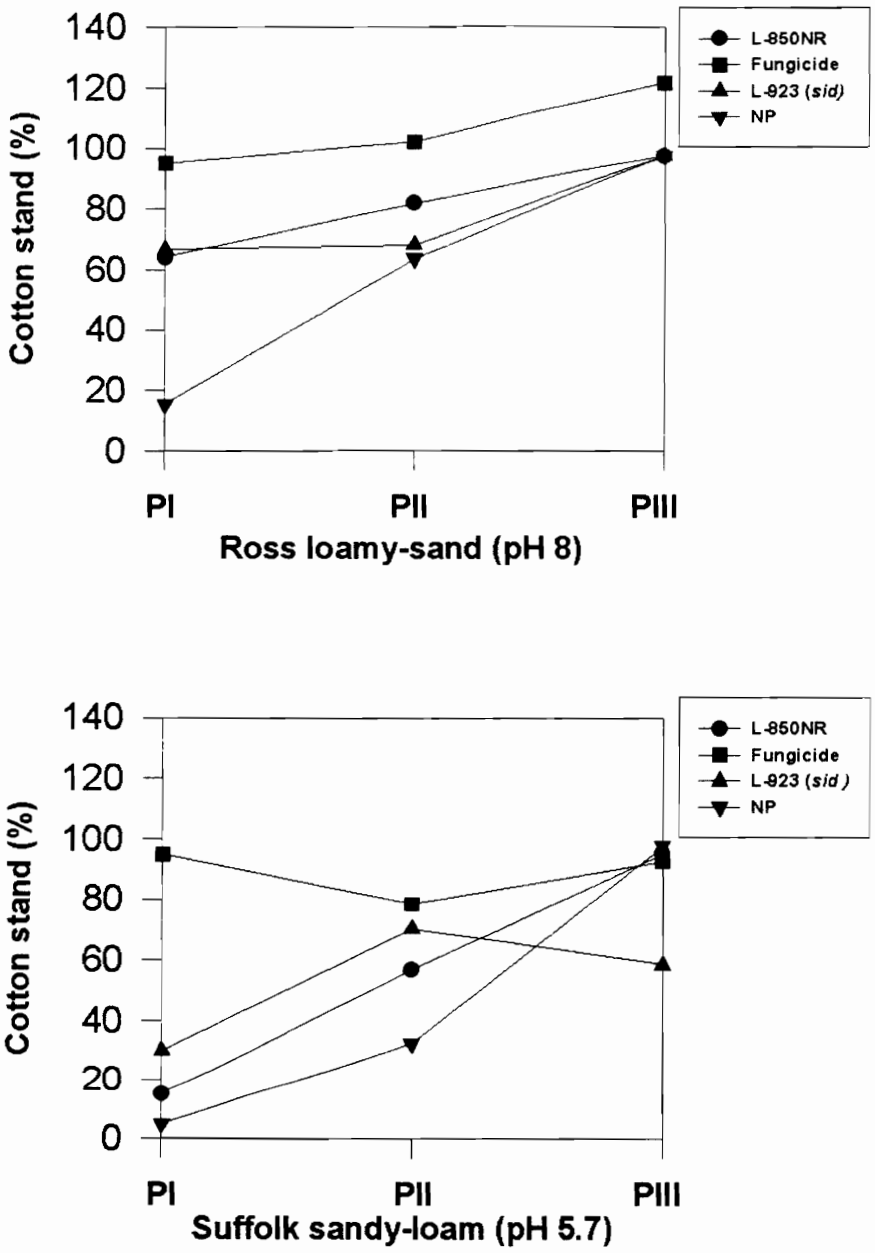

Figure 1. Final cotton stands at harvest (42 DAP) as affected by pathogen density for seed treatments *Pseudomonas chlororaphis* L-850NR, L-923 (*sid*), metalaxyl/PCNB fungicide, and the nontreated pathogen control (NP). Mean values are relative to the nontreated control. For pathogen soil inoculum levels PI (high), PII (intermediate), and PIII (low) see Table 1.

under severe pathogen pressure (PI). Treatment with L-923 (*sid*) resulted in higher final stand counts than strain L-850NR in the Suffolk soil (low, intermediate pathogen levels), indicating greater pathogen suppression with the (*sid*) mutant, and suggesting enhanced synthesis of antibiotic compounds in response to the high Fe^{+3} and/or P concentrations. This trend was not evident in the Ross soil. The delayed emergence in response to low temperatures for pathogen level PIII was again reflected in the final stand counts for L-923. Treatment with the fungicide produced a final stand count (% relative to the nontreated control) greater than 100% in the Ross soil (PIII), the cooler temperatures resulting in decreased emergence of the nontreated control as well, possibly due to increased seed colonization by deleterious saprophytic microflora.

Root rot disease severity (DSRR) and hypocotyl/tap disease severity (DSHT) (phase 2) assessed using a rating scale of 1(0% area with symptoms) to 6 (81-100%) showed no overall main effect of soil (P = 0.05) (Table 4). There was no overall main effect of pathogen with respect to root rot severity (DSRR), however, lesion severity ratings on hypocotyl/tap roots (DSHT) were significantly lower (P=0.01) at PIII (low) relative to those at PII (intermediate) and PI (high). The severity of DSHT symptoms therefore appeared to be influenced to a greater extent by changes in soil propagule density. The DSRR ratings, pooled with respect to soil and pathogen inoculum density, indicated differences (P=0.05) in root rot suppression among bacterial treatments. Plants treated with mutant M-3002 (*ant*) exhibited root rot symptoms (DSRR) equal to the nontreated pathogen

Table 4. Root rot disease severity (DSRR) and hypocotyl/tap lesion disease severity (DSHT) at harvest 42 DAP, in response to soil, inoculum densities of *P. ultimum* and *R. solani*, and seed treatment

Disease severity variable[1]	Soil[2]	Inoculum density[3]	Seed treatment[4]
Root rot severity (DSRR)	NS[5]	NS	** L-850wt 1.5 bc
			M-54 1.7 c
			L-923 1.6 c
			L-850NR 1.7 c
			M-3002 2.3 d
			NP 2.5 d
			F 1.1 a
			N 1.0 a
Hypocotyl/tap lesion severity (DSHT)	NS	*** PIII 1.8 b	**L-850wt 1.8 bc
		PII 2.1 a	M-54 2.4 cd
		PI 2.6 a	L-923 2.2 bc
			L-850NR 2.1 bc
			M-3002 3.0 d
			NP 3.0 d
			F 1.6 ab
			N 1.2 a

[1]Disease severity rating scale of 1-6 used to assess DSRR and DSHT, with 1 = 0, 2 = 1-20, 3 = 21-40, 4 = 41-60, 5 = 61-80, and 6 = 81-100% root rot or hypocotyl/tap root surface with lesions, respectively.
[2]Ross loamy-sand (pH 8.0) and Suffolk sandy-loam (pH 5.7). Main effect of soil on DSRR and DSHT.
[3]Pathogen soil densities PI (high) , PII (intermediate), and PIII (low) (see Table 1). Main effect of pathogen.
[4]L-850 = w.t. *P. chlororaphis*; L-923 = (*sid*) mutant; M-54 and M-3002 = (*ant*) mutants; F = PCNB/metalaxyl fungicide; NP = nontreated pathogen control; N = nontreated control. Mean values presented are pooled with respect to soil and pathogen.
[5]NS = Not significant.
*** (P = 0.01); ** (P = 0.05). Means with same letters do not differ significantly, (DMRT) within each individual disease variable.

control. Protection against DSRR by M-3002 was significantly less (P = 0.05) than all other bacterial treatments. The main effect of seed treatment on hypocotyl/tap lesion severity (DSHT), pooled relative to soil and pathogen inoculum density also indicated that the ratings of plants treated with M-3002 (*ant*) were equal to that of the nontreated pathogen control (Table 4). Suppression of hypocotyl/tap symptoms by L-850 (wt), L-923 (*sid*) and L-850NR was equivalent that provided by the fungicide treatment but not by treatment with the (*ant*) mutants M-54 and M-3002. No significant difference in DSRR and DSHT suppression was detected between wild-type L-850 and the spontaneous antibiotic-resistant derivative L-850NR. Hypocotyl/tap lesion severity (DSHT) ratings in general were higher than DSRR for each individual treatment, indicating less effective suppression of *Rhizoctonia* by both *P. chlororaphis* and the recommended fungicide. *In vitro* antifungal bioassays with *P. chlororaphis* have demonstrated fungistatic action against *R. solani* and fungicidal action against *P. ultimum* (data not shown). The difference in sensitivity displayed by *P. ultimum* and *R. solani* in response to secondary metabolites synthesized by *P. chlororaphis in vitro* may be reflected in the higher ratings for DSHT. DSRR and DSHT ratings were compared for several bacterial treatments (Fig 2) for PI, PII and PIII pathogen densities, irrespective of soil type. The degree of symptom suppression (for both DSRR and DSHT) was equivalent between the wild-type and L-923 (*sid*) mutant at all three pathogen inoculum densities. Suppression of root rot (DSRR) was fairly uniform across all

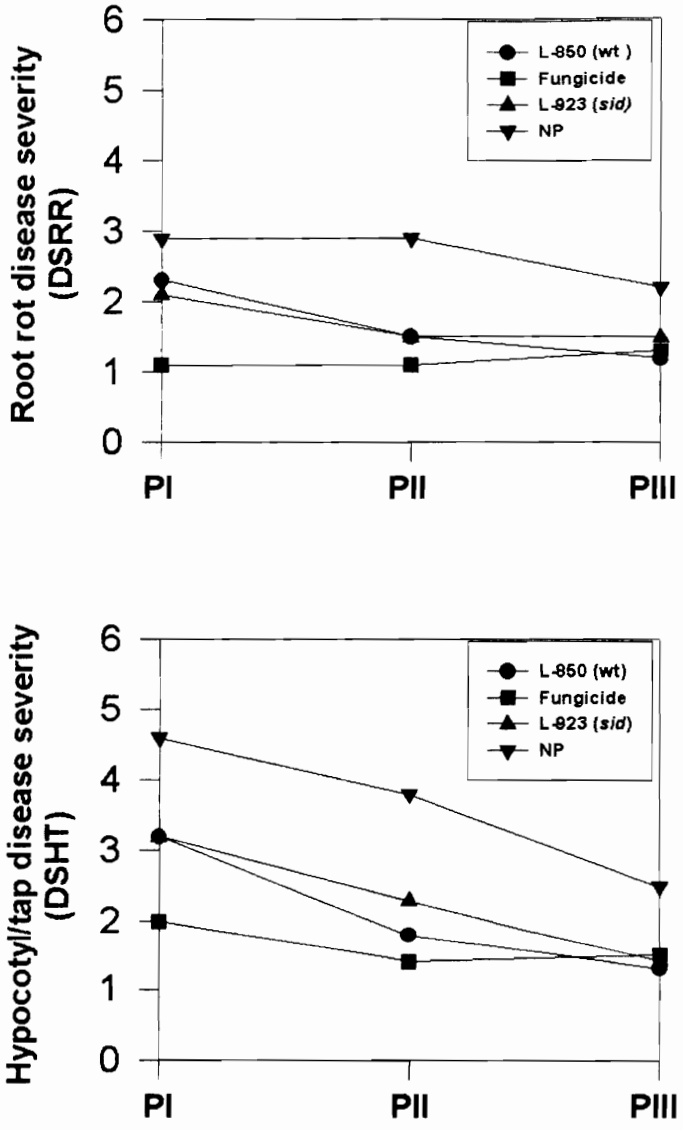

Figure 2. Comparison of *Pseudomonas chlororaphis* (L-850 wt), (*sid*) mutant L-923, metalaxyl/ PCNB fungicide, and the nontreated pathogen control (NP) treatments for suppression of DSRR and DSHT symptoms on cotton at harvest (42 DAP), in response to soil pathogen density (PI, PII, PIII). Ratings are the pooled means for both soils at each pathogen level (PI, PII, PIII). For DSRR and DSHT rating scale and PI, PII and PIII levels, see text and Table 1, respectively.

three densities, whereas suppression of hypocotyl/tap lesions (DSHT) increased with decreasing pathogen density.

Root rot disease severity ratings (DSRR), pooled relative to soil, were dependent on the soil pathogen inoculum density (Fig 3). At high (PI) and intermediate (PII) pathogen pressure, none of the bacterial strains provided suppression of root rot symptoms equivalent to the recommended fungicide, but wild-type L-850 and the (*sid*) mutant L-923 treatments were comparable to the fungicide at low pathogen pressure (PIII). Plants treated with the (*ant*) mutant M-3002, exhibited root symptoms equivalent in severity to the nontreated pathogen control plants at PI and PIII inoculum levels (P = 0.05). Wild-type L-850 and (*sid*) mutant L-923 treatments provided similar suppression of root rot symptoms (P = 0.05) at both the high (PI) and intermediate (PII) pathogen densities, although L-850 provided significantly greater control at density PIII.

A similar trend was evident for DSHT (Fig 4), although protection provided by treatment with wild-type L-850 and the (*sid*) mutant L-923 was statistically indistinguishable (P = 0.05) at all three pathogen inoculum densities. In contrast to DSRR, protection by strain L-850 was equal to the fungicide seed treatment at both the intermediate (PII) and low (PIII) pathogen densities.

An additional test in the Ross soil at intermediate (PII) pathogen pressure was not harvested at 42 DAP, but maintained in the greenhouse until 113 DAP to assess long-term survival of *P. chlororaphis* on cotton roots in pathogen-infested soil. Cotton root population density and distribution data were obtained for L-

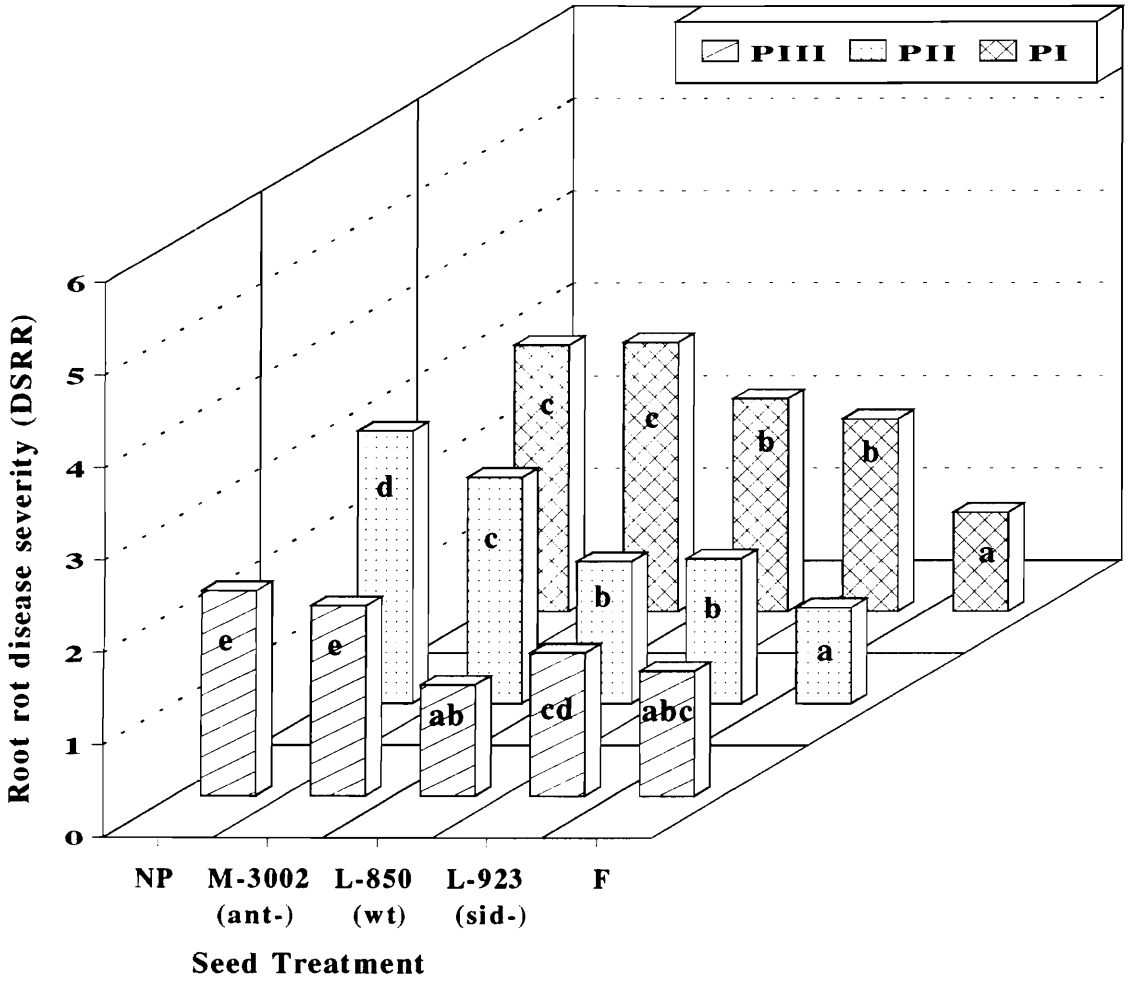

Figure 3. Comparative suppression of cotton root rot symptoms, expressed as a root rot disease severity rating at three soil pathogen densities in response to selected treatments [*P. chlororaphis* L-850wt, L-923(sid-) mutant, M-3002(ant-) mutant, metalaxyl/PCNB fungicide(F), nontreated pathogen control(NP)]. Ratings are pooled means for both soils. For root rot rating scale see text. Nontreated control rating of 1.0 not included. Bars with same letters within each pathogen level not significantly different (DMRT, P = 0.05).

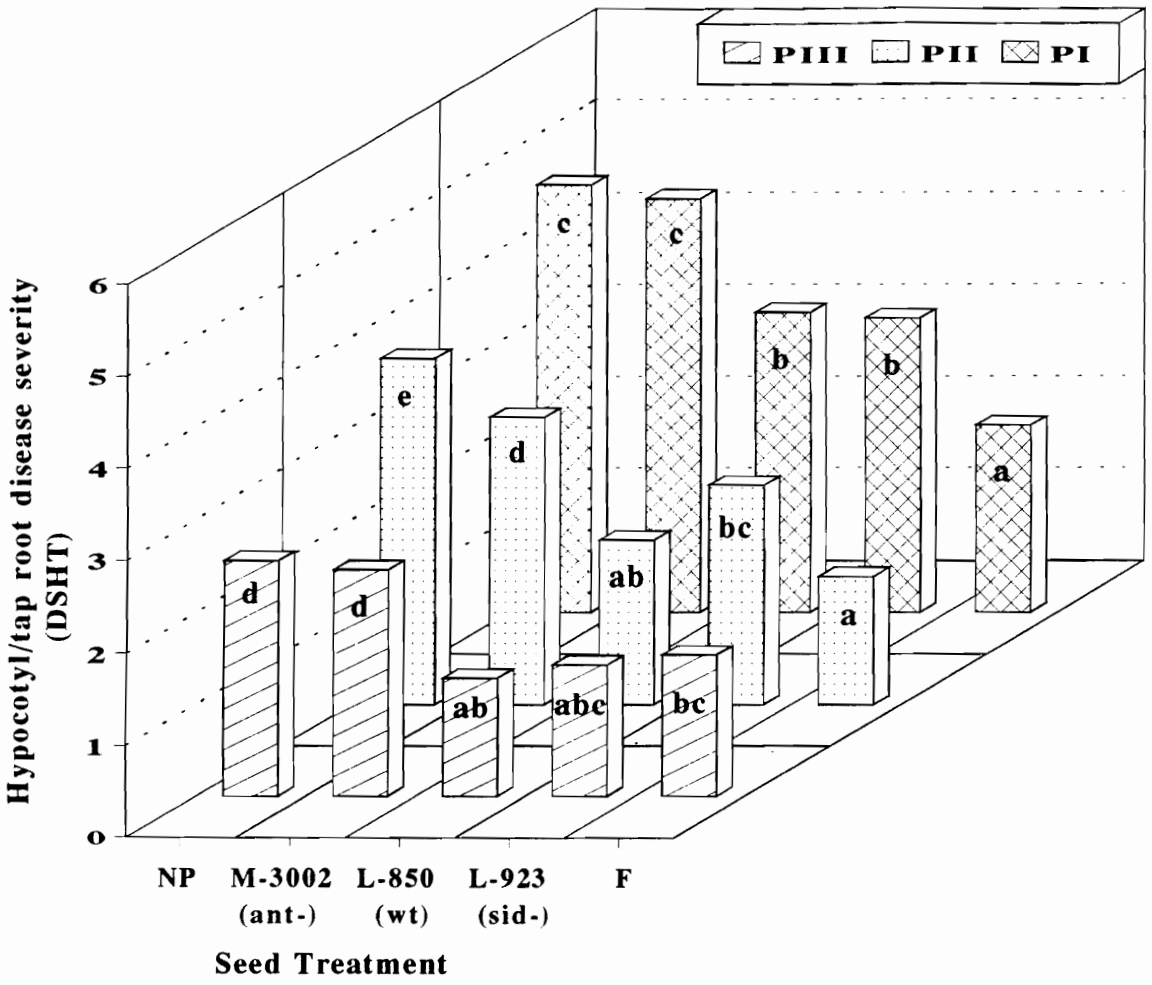

Figure 4. Comparative suppression of hypocotyl/tap root lesions, expressed as a hypocotyl/tap disease severity rating at three soil pathogen densities in response to selected treatments [*P. chlororaphis* L-850wt, L-923(sid-) mutant, M-3002(ant-) mutant, metalaxyl/PCNB fungicide(F), nontreated pathogen control(NP)]. Ratings are pooled means for both soils. For root rot rating scale see text. Nontreated control rating of 1.0 not included. Bars with same letters within each pathogen level not significantly different (DMRT, P = 0.05).

850NR, L-923 (*sid*), and the (*ant*) mutants M-54 and M-3002. Populations of L-923 (*sid*) were detected at densities between log 4.5 and log 5.1 cfu/cm^2 on 3 of 4 root sections, as were populations of M-3002 (*ant*) (data not shown). Densities were slightly lower for M-54 (*ant*) and L-850NR (log 3.8 - 5.0). No bacterial populations were recovered on lower tap roots, or numbers were below the detection limits of dilution plating. The majority of lower tap roots 113 DAP were girdled by *R. solani* lesions. Population values were not obtained for the Suffolk soil.

Loss of cotton root area in response to pathogen inoculum density. Reductions in cotton root area due to root rot and/or progressive root pruning by *P. ultimum* and *R. solani* were calculated (TIA software program) using area values for the nontreated (N) and nontreated pathogen (NP) control plants obtained at harvest (42 DAP). Although both soils were infested with equivalent levels of pathogen propagules, inoculum densities produced dissimilar reductions in root area (Table 5). At pathogen level PI (high), the mean reduction in cotton root area was 64 and 60%, and at level PII (intermediate), 33 and 12%, respectively, for the Ross and Suffolk soils. Reductions were significant at the 1% level for PI (high) and PII (intermediate) in the Ross soil, and at the 5% level for PI (high) in the Suffolk soil. A 16% stimulation in root area was observed with the nontreated pathogen control plant roots exposed to PIII (low) conditions in the Suffolk soil (negative % value), a phenomenon not observed in the Ross soil.

Table 5. Loss of cotton root area (%) in response to soil and pathogen inoculum density

	% root area reduction[2]	
Pathogen density[1]	Ross loamy-sand pH 8.0	Suffolk sandy-loam pH 5.7
PI	64.5 **	60.6 *
PII	33.2 **	12.2
PIII	10.7	(-)16.5[3]

** (P = 0.01); * (P = 0.05).

[1]For pathogen soil inoculum densities, see Table 1.

[2]% reductions calculated using mean root area values of nontreated pathogen control and nontreated control plants. Data with asterisks are significantly different, ($P \leq 0.05$) according to t-tests.

[3]Stimulation in root area for nontreated pathogen control plants (mean values of 13.26 and 15.89 cm^2 for nontreated and nontreated pathogen controls, respectively.

Discussion

The level of *P. ultimum* and *R. solani* suppression by *P. chlororaphis* differed between mutant and wild-type strains and was dependent on soil pathogen propagule density, the disease phase (phase 1 seedling pre- and postemergence damping-off vs phase 2 hypocotyl/root rot of young plants) and to a slight extent, soil physical/chemical characteristics. Differences in response to soil might have been more statistically significant had there been a greater number of treatment replicates. Generally, differences in suppressiveness among bacterial strains were more evident for phase 2 of the disease complex. Loss of cotton root area was also dependent on soil physical/chemical characteristics and more critically, on pathogen inoculum density.

Wild-type L-850, M-54 (*ant*) and L-923 (*sid*) seed treatments produced higher mean growth responses for all variables measured over those of both the nontreated pathogen control (NP) and nontreated control plants (N). These data, together with rhizosphere colonization results (Chapter IV) provide evidence in support of root and foliar growth promotion of cotton both in the presence and absence of *P. ultimum* and *R. solani*.

Notably, the largest improvement in stand emergence by bacterial treatment relative to the nontreated pathogen control was demonstrated at the highest level of pathogen pressure (PI). Stand improvements due to bacterial treatment in general decreased in magnitude with decreasing inoculum density. Although pre- and postemergence damping-off suppression is a key factor in stand

establishment, moderate stand losses may not result in yield reductions, as more rapid growth of remaining plants under less crowded conditions may compensate for initial stand losses (1). Evidence in support of this was obtained by Hagedorn et al. (6) where two *P. fluorescens* strains applied to cotton seed did not provide increased seedling stands, but produced cotton yields equivalent to strains that did. In this context, protection provided by biocontrol strains against the progressive root pruning of lateral roots and stem girdling of young plants during the early part of the season can have an impact on cotton yields.

Protection against this phase of the disease complex (phase 2) was demonstrated by the root rot (DSRR) and hypocotyl/tap lesion (DSHT) severity ratings at 42 DAP. A mean root rot rating (DSRR) of 1.5 was achieved by treatment with wild-type L-850, representing the pooled value of all three pathogen densities and both soil types. This indicated that suppression of root rot by the wild-type strain was provided regardless of soil physical/chemical characteristics. Control comparable to L-850 (wt) was provided by the (*sid*) mutant L-923 (pooled DSRR rating = 1.6) which is (ant^+). An equivalent trend for these two strains was demonstrated for hypocotyl/tap root lesion symptom repression (1.8 and 2.2) providing evidence in support of antibiotic production as a biocontrol mechanism. Treatment with mutant M-3002 (ant^-, sid^+), produced consistently higher root rot (DSRR) and hypocotyl/tap lesion (DSHT) severity ratings in both soils than either the (*sid*) mutant L-923, or wild-type L-850, further supporting a role for antibiotic synthesis. The decreased suppressive activity by M-3002 (*ant*) in the present

study may be related to the decrease in rhizosphere competence observed with this mutant (Chapter IV). Residual biocontrol activity was evident with (*ant*) mutant M-54, indicating the production of more than one antifungal antibiotic acting in the suppression of disease phases 1 and 2 induced by *P. ultimum* and *R. solani*.

In vitro agar-based bioassays investigating the effect of iron (Fe^{+3}) and phosphorus (P) on antifungal activity by wild-type strain L-850 resulted in 100% inhibition of *P. ultimum* and *R. solani* mycelial growth over the range of 0-100 µM Fe^{+3} and 0-1 mM P, although concentrations of P > 1 mM decreased inhibitory activity by only 10 to 20%. This range most likely represents the P and Fe^{+3} optima for synthesis of multiple secondary metabolites, as strain L-850 (wt) produces siderophores, and other uncharacterized antifungal metabolites, of which one or more phenazines are included. Secondary metabolite biosynthesis in general has been found to be responsive to low P concentration (15). High P inhibited phenazine-1-carboxylic acid (PCA) by *P. aureofaciens* (now *P. chlororaphis*) (24). The effect of Fe^{+3} has not been as well characterized and can vary depending on the secondary metabolite, *e.g.*, low iron stimulated siderophore production but repressed pyocyanin (15) and PCA (23) accumulation. Given the wide range of mineral optima established by *in vitro* assays, repression of *P. ultimum* and *R. solani* by *P. chlororaphis* L-850 should be comparable in the two soil types due to one or more antifungal secondary metabolites (siderophores and antibiotics in the Ross soil, antibiotics in the Suffolk soil) synthesized in response to the Fe^{+3} and P levels in both soils. Although not significant, DSRR and DSHT

ratings (disease phase 2) were higher in the Suffolk soil (pH 5.7, high P, high Fe^{+3}) for both wild-type and mutant strains, indicating; 1) physical/chemical characteristics of the Suffolk soil were less favorable to growth of the bacterial strains compared to the fungal pathogens or 2) physical/chemical characteristics were less favorable for synthesis of secondary metabolites inhibitory to the pathogens. Evidence in support of the former was provided previously (Chapter IV) where bacterial population densities in general were 1/8 to 3/4 log unit lower on cotton roots grown in the Suffolk soil compared to the Ross soil. The generation and testing of mutants of strain L-850 deficient in siderophores or antibiotics demonstrated similar suppressiveness between L-850 (wt) and L-923 (*sid*) and reduced suppressiveness with M-3002 (*ant*), defining a role for antibiotics under a wide range of soil iron and phosphorus mineral concentrations.

Siderophore production contributed to the antifungal activity of *P. fluorescens* 3551 against *Pythium* damping-off of cotton, but was not solely responsible for suppression (16), and played no role in the biological control of cucumber damping-off (14). Differences in suppression observed with the two plant hosts may have included longer emergence time (7-14 days) for cotton relative to cucumber (3-5 days), providing a greater opportunity for iron competition between the pathogen and the biocontrol strain (14). The delay in seedling emergence experienced in the present study in response to a short period of cooler greenhouse temperatures slightly enhanced phase 1 but not phase 2 biological control by strains M-54 and M-3002 at the lowest pathogen inoculum density PIII

(>100% emergence relative to nontreated control), both of which are wild-type for siderophore biosynthesis. However, the low pathogen pressure for PIII should be taken into consideration prior to making conclusions regarding the effect of siderophore and antibiotic synthesis in biological control. Disease incidence (% emergence), final stand counts, and disease severity ratings for mutant and wild-type strains of *P. chlororaphis* support the conclusions of Loper (16) and Kraus and Loper (14) who claimed a minimal role for siderophore-mediated iron deprivation as a biocontrol mechanism of seedling damping-off except under environmental conditions that delay seed germination.

The level of pathogen control expressed by strains L-923 *(sid)* and M-3002 (*ant*) during disease phases 1 and 2, supports a role for antibiotic secondary metabolite(s) production in the biofungicidal activity of *P. chlororaphis*. An additional proposed suppressive mechanism, physical exclusion and/or competition for nutrients, (14) was not investigated in the present study. Subsequent research should focus on the "streamlining" or deleting of genes involved in the biosynthesis of secondary metabolites that play a minor role or are ineffective in pathogen repression by superior rhizosphere competent strains. This approach would alleviate the waste of metabolic energy expended in synthesis of multiple antifungal compounds. Finally, the results indicate the importance of assessing pathogen inoculum density reflected in disease incidence/severity in the field situation, prior to implementing a control strategy that utilizes biological components.

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VI.

SUMMARY AND FUTURE RESEARCH APPROACHES

Summary and Conclusions

Pythium ultimum and *Rhizoctonia solani* are two soilborne fungal pathogens primarily responsible for seedling damping-off and root rot of a wide range of agronomic crops worldwide. In cotton, diseases caused by these pathogens are referred to as cotton seedling disease complex which can be subdivided into two component phases. Phase 1 refers to pre- and postemergence damping-off and phase 2 refers to the hypocotyl/root rot and lesion symptoms evident on both young seedlings and more mature plants. Seedling disease often substantially reduces root growth and has resulted in considerable losses in crop yield, averaging 3.5% annually on cotton over the past 25 years.

Rhizosphere-inhabiting pseudomonads comprise a diverse group of bacteria involved in both biological disease control of *P. ultimum* and *R. solani* and crop growth promotion. Among many mechanisms critical to biological control, two in particular, rhizosphere competence (Chapter IV), and the production of antifungal siderophores (sid) and antibiotics (ant) (Chapter V), were evaluated for *Pseudomonas chlororaphis* strain L-850 in these studies. Previous methodologies generally employed to assess root colonization have suffered from inherent difficulties with respect to expression of bacterial cfu's on a per unit basis, *e.g.*, root length, root dry weight or fresh weight. These units, intended as an estimate of the root surface area available for colonization, did not take into account

changes in root diameter as roots mature. This difficulty was circumvented by the development of the TIA software program (Chapter III) that allowed expression of bacterial populations as a function of cotton root surface area (cm^2). The TIA program permitted rapid processing of a large number of root samples and increased sample reproducibility. This powerful technique allowed repetitive assessment of reductions in cotton root area in response to high, intermediate, and low pathogen soil inoculum density, not previously addressed in the literature. Use of this program also provided data on cotton root growth in response to treatment with various *Pseudomonas* strains (mutant, wild-type) in both the absence (Chapter IV) and presence of *P. ultimum* and *R. solani* (Chapter V). Significant reductions in root area were evident between plants exposed to pathogen-free and pathogen-alone conditions in the Ross loamy sand (pH 8) for high (PI) and intermediate (PII) soil inoculum levels but not at low (PIII) propagule densities. For the Suffolk soil (pH 5.7), significant reductions in root area occurred only at high (PI) inoculum densities, indicating an influence of soil physical/chemical characteristics on the severity of phase 2 root rot and root lesion symptoms. Seed treatment with *P. chlororaphis* (mutants or wt) provided significant root growth promotion relative to the nontreated control plants even in the absence of root pathogens. The largest increase in root growth was provided by treatment with L-923 (*sid*).

In situ rhizosphere competence assays identified *P. chlororaphis* strain L-850 as a single superior root colonizing isolate, that produced siderophores and several antifungal antibiotics, including one or more phenazine compounds. The

contribution of siderophores and antibiotics to biological control of seedling disease by pseudomonads in general is host plant dependent. Paulitz and Loper (8) failed to identify a role for siderophores in the biological control of *Pythium* on cucumber, however, studies with cotton have proven inconclusive with respect to the contribution of siderophores versus antibiotics (4). Data from the present study, using mutants of *P. chlororaphis* strain L-850, provided insight into the role of antibiotics and siderophores in rhizosphere competence and in pathogen suppression on cotton.

Root colonization studies in two soils, selected for acid and alkaline pH values, as well as low and high iron and phosphorus levels, produced several important results. The bacterial strains (mutants and wild-type) migrated from the application point in a linear manner to the lower tap and lower lateral roots in the Suffolk sandy loam (pH 5.7) compared to more lateral migration (and at higher cell densities) in the Ross loamy sand (pH 8.0). Populations of all strains migrated with the growing roots in the absence of soil irrigation and persisted after day 22 at levels between log 4 and log 6 cfu/cm^2, varying between strain and root section even as total root area increased 122% between day 22 and 50. Moreover, populations in this range were evident on lower tap and lower lateral roots which also exhibited the highest populations of competitive indigenous microbes.

The loss of antibiotic production by mutant M-3002 decreased the rhizosphere competence of *P. chlororaphis*. In contrast, the loss of siderophore production (L-923) appeared to enhance rhizosphere competence. With respect to biocontrol

efficacy, differences among bacterial treatments were more evident for phase 2 than phase 1 of the disease complex. Phase 2 protection provided by L-923 (*sid*) was equivalent to the wild-type strain in both soils whereas, the level of protection decreased by treatment with M-3002 (*ant*). The similar suppressiveness demonstrated between L-850 (wt) and L-923 (*sid*) defined a role for antibiotics under a wide range of soil pH and iron and phosphorus concentrations. The research herein also demonstrated a correlation between rhizosphere competence and biological control efficacy. Greenhouse trials demonstrated a minimal role for siderophore biosynthesis in the biological control of cotton seedling disease caused by *P. ultimum* and *R. solani.* Siderophore biosynthesis may play a role in biological control under environmental conditions that result in a delay in cotton germination, such as cooler soil temperatures. Conditions that delay seed germination may allow a greater interaction period between the pathogen and the biocontrol strain making competition for limited soil iron more probable.

Future Research Approaches

Recent data (6, 9) indicate that antibiotics play a role not only in pathogen inhibition and microbial competition in the rhizosphere, but contribute to survival of the producing strain as well. The generation of an antibiotic overproducing bacterial strain would seem an attractive research goal, however, several laboratories have presented data indicating that antibiotic overproduction resulted in a shorter life span of the organism (11) or a greatly increased generation time (3). Future work, as was suggested in the present study (Discussion, Chapter V),

would benefit from "metabolic streamlining" , i.e., the deleting of genes specific to the biosynthesis of secondary metabolites that are unimportant or ineffective in the biological control of a particular host-pathogen system. A single rhizosphere competent bacterial isolate capable of synthesis of 1 to 2 secondary metabolites could be genetically modified to produce a series of derivative strains adapted to cold temperature stress, heat stress, drought stress, osmotic stress, etc. A strategy utilizing these derivatives in combination would ensure biocontrol under a wide range of environmental conditions, as would be encountered in the field. This approach, however, would not be feasible against soilborne pathogens requiring multiple antifungal compounds for suppression. A control strategy for the latter would require the use of several biocontrol isolates each of which would be modified genetically for diverse environmental conditions as above. The genetic modifications and screenings involved in this approach would be a time consuming and difficult task. Although the chemical structures and biosynthetic pathways of many antibiotics produced by pseudomonads such as pyrrolnitrin, phenazine-1-carboxylic acid and pyoluteorin were elucidated in the late 1950's and early 60's, little is known about the response regulator proteins, and sensor and activator molecules involved in the biosynthesis of the majority of antibiotic compounds. Only recently, a genomic region was identified as necessary for synthesis of pyoluteorin (2) and a genomic region capable of conferring pyrrolnitrin synthesis (and thus antifungal activity) was cloned from *P. fluorescens* and transferred to a non-producing *P. fluorescens* strain (1). Little is also known of the

specific nutritional factors in the root and soil environment that influence production of antibiotics. With the advent of sensitive transcriptional reporter systems such as *inaZ* which is a promotorless ice nucleation activity gene, the effect of specific soil mineral concentrations or carbon/nitrogen sources on transcription (ice nucleation activity) may be assessed if a chemically responsive promoter exists for the antibiotic of interest (5). In this context, the response of antibiotic compounds to specific water soluble and volatile host root exudates could also be determined. Although there is a fairly comprehensive understanding of the types of molecules present in root exudates (7), little is known about the utilization of these exudates for bacterial growth or their effect on secondary metabolite production by bacterial biocontrol strains.

The research approaches outlined thus far have focused on the bacterial biocontrol agents, however, there is a need for greater understanding of the interactions between the host plant and the bacterium. Knowledge of the specific root exudates of a particular host may lead to genetic engineering of the biocontrol strain for enhanced utilization of select compounds. Such studies have been recently initiated by Thomson et al. (10) where a levanase gene transferred to *Pseudomonas* conferred on the isolate the ability to utilize sucrose as the sole carbon source, enabling the isolate to grow in the interior of sugar cane. Finally, though the transfer of bacterial genes involved in secondary metabolite synthesis to the host plant appears to be both an obvious and attractive research direction, progress has evolved slowly because transformation procedures for many

agronomically important crops have only recently been developed. Such techniques require a large input of time and expense for a single crop plant and many genes of bacterial origin are unlikely to be efficiently expressed in plant systems (10).

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VII

APPENDICES

CONTENTS

Table 12. Influence of soil and time (22, 36, 50 DAP) on the mean population density and distribution patterns of wildtype L-850NR, L-923 (*sid*), and M-3002 (*ant*) on four cotton root sections (UT, UL, LT, LL)

Ross loamy-sand (pH 8.0)									
	L-850 NR			L-923			M-3002		
Root	22	36	50	22	36	50	22	36	50
UT[1]	6.1± 0.1[2]	6.5 ± 0.03	6.4 ± 0.02	6.2 ± 0.01	6.7 ± 0.1	6.2± 0.3	6.2 ± 0.1	6.3 ± 0.02	6.2 ± 0.09
UL	5.2 ± 0.03	5.3 ± 0.3	5.0 ± 0.3	6.0 ± 0.4	5.6 ± 0.05	5.4 ± 0.1	5.6± 0.1	5.3 ± 0.05	4.9 ± 0.1
LT	6.0 ± 0.6	5.0 ± 0.1	3.8 ± 0.09	5.6 ± 0.1	5.5 ± 0.3	4.9 ± 0.2	5.6 ± 0.08	4.6 ± 0.6	4.8 ± 0.2
LL	5.0 ± 0.4	4.6 ± 0.1	3.9 ± 0.2	4.9 ± 0.2	5.2 ± 0.2	5.0 ± 0.1	5.2 ± 0.08	4.7 ± 0.03	4.1 ± 0.1
Suffolk sandy-loam (pH 5.7)									
UT	5.6 ± 0.4	6.2 ± 0.2	6.1 ± 0.4	5.2 ± 0.2	6.1 ± 0.09	5.8 ± 0.3	4.7 ± 0.8	5.6 ± 0.2	5.5 ± 0.2
UL	4.7 ± 0.4	5.2 ± 0.02	4.5 ± 0.2	5.0 ± 0.5	5.5 ± 0.1	3.8 ± 0.2	4.5 ± 0.2	4.9 ± 0.1	4.0 ± 0.3
LT	4.9 ± 0.2	4.1 ± 0.3	4.0 ± 0.5	5.5 ± 0.3	5.6 ± 0.2	6.0 ± 0.3	4.9 ± 0.1	5.4 ± 0.4	4.6 ± 0.2
LL	4.5 ± 0.3	4.0 ± 0.4	4.4 ± 0.2	4.8 ± 0.2	5.0 ± 0.3	4.7 ± 0.4	3.8 ± 0.2	4.6 ± 0.1	3.5 ± 0.3

[1]UT=upper tap root, LT=lower tap, UL=upper lateral roots, LL=lower lateral roots.
[2]Data presented are the population means (± S.D.) of three replicates/bacterial treatment.

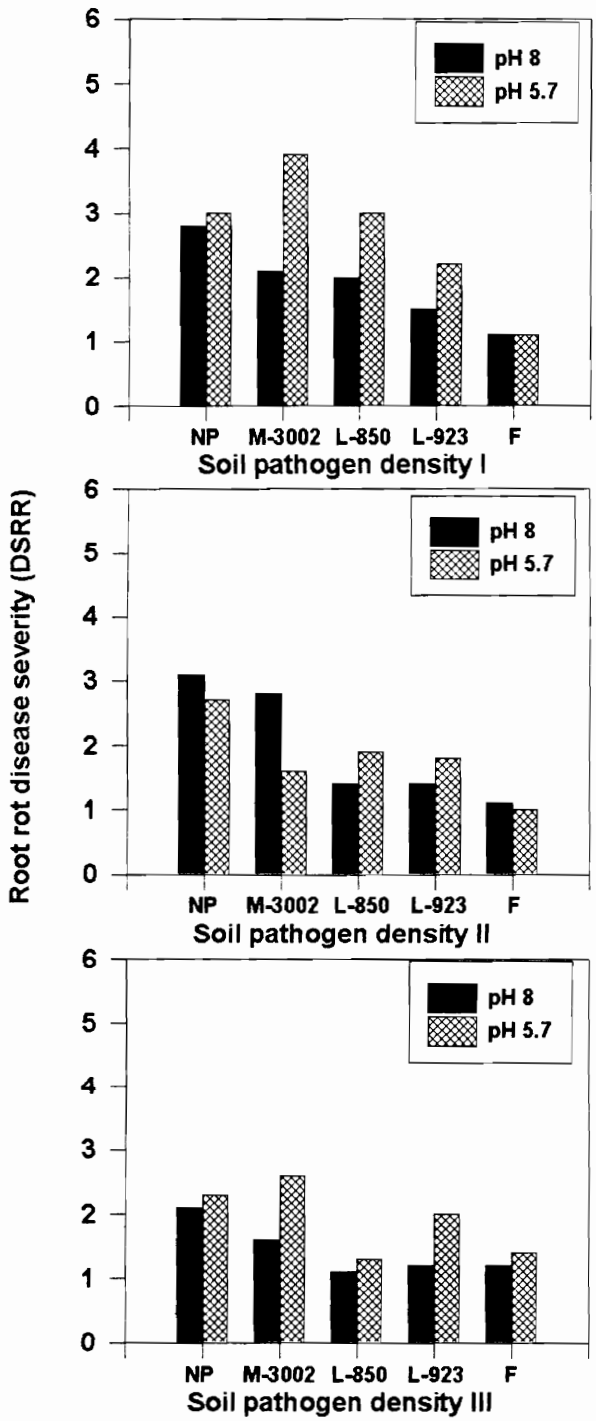


Figure 14. Comparative suppression of cotton root rot symptoms, expressed as a root rot disease severity rating, in two soils(pH 8, 5.7), each at three soil pathogen densities. The treatments compared included wildtype *Pseudomonas chlororaphis* (L-850), mutantsL-923 (*sid*) and M-3002 (*ant*), PCNB/metalaxyl fungicide (F), and the nontreated pathogen control (NP). For root rot disease severity rating scale see Chapter V. Mean ratings presented are unpooled with respect to soil pH.

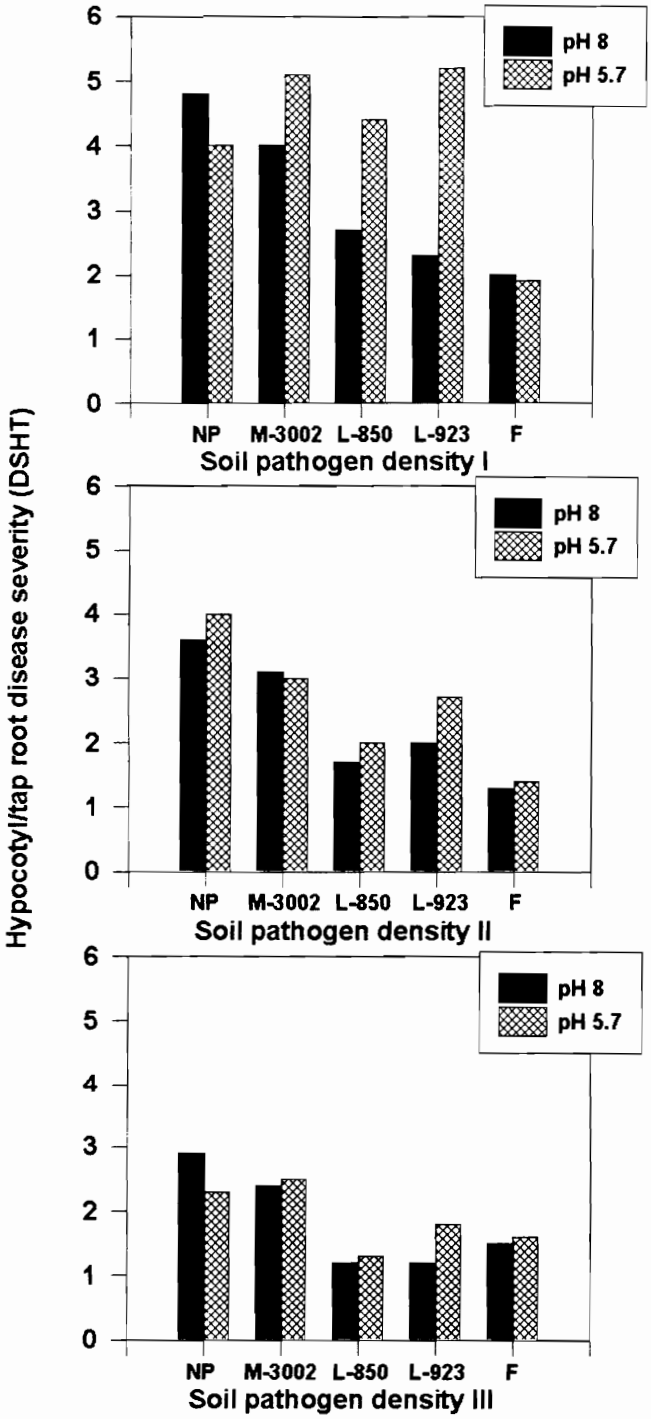


Figure 15. Comparative suppression of hypocotyl/tap root lesion severity in two soils (pH 8, 5.7), each at three soil pathogen densities. The treatments compared included wildtype *P. chlororaphis* (L-850), mutants L-923(*sid*) and M-3002 (*ant*), PCNB/metalaxyl fungicide (F), and a nontreated pathogen control (NP). For hypocotyl/tap lesion severity rating scale see Chapter V. Mean ratings presented are unpooled with respect to soil pH.

APPENDIX A

DNA Isolation from Bacterial Cells
Modification of the Marmur Method

The Marmur procedure was the first widely used method for the isolation of DNA from bacteria and other organisms. The following procedure is a variation of the original protocol. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Molec. Biol. 3:208-218.

Cell Suspension Buffer:

10 mM Tris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

0.35 M Sucrose

If the cells are going to be physically disrupted (passage through a French pressure cell or by shaking with glass beads), there is no need to include the sucrose in the suspension buffer.

Lysing Solution:

	2X
100 mM	Tris-HCl (pH 8.0)
0.3 M	NaCl
20 mM	EDTA
2%	Sodium Dodecylsulfate (SDS)
2%	2-mercaptoethanol
100 µg/ml	Proteinase K

Add Proteinase K (125 µl of a 20 mg/ml stock solution per 25 ml of 2X lysing solution) and 2-mercaptoethanol (2% final concentration) to the amount of 2X lysing solution needed (do not keep this mixture longer than one day).

Other Solutions and Buffer:

5 M Na_2ClO_4, 700 g/l in water

Isopropyl alcohol (IPA)

76% Ethanol (80% ethanol-water, using 95% stock)

3 M sodium acetate (pH 6.0)

Tris-EDTA buffer (TE): 10 mM Tris-HCl, 1 mM EDTA (pH 8.0)

Chloroform ($CHCl_3$): Chloroform-isopentanol (25:1 v/v)

Phenol-Chloroform: A v/v mixture of water saturated phenol and chloroform-isopentanol, containing 0.1% 8-hydroxyquinoline.

RNase mixture: Prepare a 1 mg/ml solution of RNase A in 10 mM Tris, 15 mM NaCl (pH 7.5). Heat at 80 C for 20 min. to inactivate any contaminating DNases. It can then be stored at -20 C until used. To the amount of RNase A needed for a DNA isolation, add T^1 RNase to a concentration of 4,000 units/ml (it usually comes as 400-600 units/µl).

PROCEDURE

The procedure as written is for 300 to 600 ml of culture. The cell suspension and lysing solution volumes can be scaled down for smaller volume or low density cultures and increased for larger cultures.

1. Pellet the cells by centrifugation. After decanting the spent medium, let the bottles stand upside down on paper towels so that the remaining few ml of medium can drain. Resuspend the harvested cells in a volume of 25 ml for the French pressure cell or glass bead disruption (that is the capacity of the pressure cell and shaking bottle).

   Note: An easy procedure for resuspending the cells is to add part of the buffer to each centrifuge bottle (but not over 15 ml), stir the pellet a bit with a glass stirring rod, and than shake the bottle on a reciprocal shaker for 5 to 10 min. Disrupt any residual clumps by drawing up and expelling

them, using a 10 ml pipette fitted with a rubber bulb. Cell clumps will not lyse properly. Then transfer the cells to the flask.

2. Digest the cell walls with lytic enzyme for 1 hour.

   2A. **Gram-negative bacteria:** Add dry lysozyme (about 1/8 t) and incubate the cell suspension, which may range from 5 to 10 min at room temperature to an extended period of time at 37 C, depending on the cell wall characteristics of the group of organisms with which one is working. Remove small samples of the cell suspension (100 µl) and mix with an equal amount of the 2X lysing solution. If the cells lyse, go to step 3, if not, continue the incubation (place the cell suspension in a 37 C incubator to increase the lysozyme activity.

3. Mix a volume of the 2X lysing solution that is equal to the cell suspension and a volume of 5 M Na_2ClO_4 that is equal to 1/4 of the combined total. This mixture will form a precipitate, warm it to about 50 C to get back into solution. When the cells are susceptible to lysis, mix this lysing mixture with the cell suspension with a quick swirling action; so that the cells and lysing solution are uniformly mixed before the cells lyse. Lysis is evident by the changing of the cell suspension from a turbid suspension to a translucent viscous solution. Incubate the lysate for about 4 hr at 50 to 60 C to degrade cellular proteins that may be associated with the DNA (the time is arbitrary, for some tissues we let the incubation go overnight, if you find that 1 or 2 hrs works as well as 4, fine).

4. Add 15 ml of phenol-chloroform, shake by hand to make sure that the phenol-$CHCl_3$ is uniformly homogenized with the lysate, then shake on wrist action shaker for 20 min. Transfer to 50 ml centrifuge tubes and centrifuge at 12,000 rpm for 10 min. Rinse the flask and let it drain during the centrifugation time.

Slowly decant as much of the upper aqueous layer back into the flask as you can without getting any of the phenol-$CHCl_3$ over. Remove the rest of the aqueous layer with an inverted 5 ml pipette attached to a pipetting device. Again add 15 ml of $CHCl_3$, shake and centrifuge again. If there is still a protein layer, repeat the phenol-$CHCl_3$ extraction a third time.

5. Carefully decant and/or remove the lysate from the final phenol-$CHCl_3$ extraction to a 125 ml Erlenmyer flask (taring the flask on a top loading balance works well for estimating the final volume). Add isopropanol, equal to 0.6 volume of the lysate, and swirl to mix. The DNA will precipitate to form a loose clot (this will not be the case if the cells were physically disrupted). Hold the DNA clot back with a Pasteur pipette, and pour the rest of the lysate-isopropanol mixture out. Add about 25 ml of the 76% ethanol. Let stand for 10 to 15 min with occasional swirling. Pour off the this alcohol mixture and repeat the washing with another 25 ml. Decant the ethanol-water such that the DNA clot remains on the bottom of the flask (it will stick quite firmly to the flask). Stand the flask upside down to drain completely, then place in a 37 C incubator to dry the DNA. If a clot did not form when the isopropanol was added (which will be the case with physically disrupted cells), the precipitate will have to be collected by low speed centrifugation (5-7,000 rpm).

6. Dissolve the DNA in 20 ml of TE buffer, add 0.25 ml of RNase mix, and incubate at 37 C for 1 hr. Extract one time with 5 ml of chloroform-isopentanol, centrifuge, and save the aqueous layer.

7. Add 0.1 volume (2 ml) of 3 M sodium acetate, mix, and place into a beaker (100 ml). Overlay with 2 volumes of 95% ethanol and collect the DNA on a glass stirring rod "spool" by simultaneously stirring and spinning the rod. After all of the DNA has been collected (the lysate and ethanol mixed to a single

phase), pour the liquid out, and gently press the glass rod against the side of the beaker to squeeze out as much of the liquid as possible. Then pour 10 to 20 ml of 76% ethanol-water mixture into the beaker to wash the precipitated DNA. Place the stirring rod (inverted) in a test tube rack to dry.

8. Dissolve the DNA in 3 to 5 ml of TE (the DNA should be at a concentration of at least 0.5 mg/ml). Store at -20 C.

APPENDIX B

Filter Paper Agar Diffusion Bioassay - Time Course Study of Antifungal Metabolite Production

Purpose: To study the effect of iron (Fe^{+3}) and phosphorus (P) concentrations on antifungal compounds released from viable cells of *P. chlororaphis* L-850 over time (24-144 hours incubation).

Protocols:

1. Bacterial inoculum preparation.

A -80 C stock culture aliquot of strain L-850 was grown overnight in mRSM medium supplemented with iron (100 µM) in the form of $FeCl_3$ until a turbidity of 0.9 (A_{640}). Bacterial cells were centrifuged, washed twice in distilled water and resuspended in same. The turbidity of the final solution was adjusted to 0.7 (A_{640}).

2. Assay medium.

Modified RSM agar medium (pH 7.0) was ammended with 0 to 200 µM Fe^{+3} (supplied as $FeCl_3$) and 0 to 100 mM phosphorus (supplied as KH_2PO_4, pH 7.0). Phosphorus concentration was 1 mM P for the iron assay and 100 µM Fe^{+3} for the P assay. Aliquots from P and Fe stock solutions were added to mRSM after autoclaving.

3. Fungal inoculum.

Potato dextrose agar (PDA; Difco, Detroit MI) was prepared according to manufacturer's instructions. PDA plates were inoculated with a single plug (4 mm) of each test fungus and incubated for 24 hours at 25 C.

4. Agar diffusion assay.

A 0.22 µ Supor® 200 47 mm membrane filter (Fisher Scientific; Norcross, GA) was placed in the center of each agar plate followed by application of 200 µl bacterial suspension to the filter. Plates were incubated at 28 C for 24, 48, 72, 96, 120 and 144 hours to assess synthesis and subsequent diffusion of antifungal substances into the agar medium below.

Following each incubation interval, the filter suspension was discarded, and a 4 mm plug from the perimeter of a 24 hour *P. ultimum* or *R. solani* culture was placed in the center of the plate. A control treatment consisted of 200 µl distilled water prior to fungal inoculation.

5. Data collection.

Mycelial growth (mm) was recorded after 24 and 48 hours incubation for each time-treatment concentration (control growth after 48 hours had reached the perimeter of the 60 mm petri plate). Growth inhibition (%) of the fungi was calculated relative to control growth values. Each time-treatment concentration was replicated twice per test fungus and the experiment repeated once.

Results:

a. *Pythium ultimum* - Inhibition of *P. ultimum* was 100% at all concentrations of Fe^{+3} up to and including 120 hours bacterial incubation measured after 24 hours fungal incubation (Fig. 16). Percent inhibition was also 100% up to and including 144 hours incubation for 0 and 1 mM P. The compounds that diffused into the agar were fungicidal at 24 hours bacterial incubation as no growth occurred when fungal plugs were transferred to fresh PDA plates, and fungistatic at 48, 72, 96, 120 and 144 hours bacterial incubation.

b. *Rhizoctonia solani* - Inhibition of *R. solani* was 100% at 24 and 48 hours bacterial incubation measured after 24 hours fungal incubation at all Fe^{+3} levels and at 0 and 1 mM P (Fig. 17). Following 48 hours bacterial incubation, inhibition ranged from 70 to 92%. Fungicidal and fungistatic observations were as for *P. ultimum*.

Growth inhibition measured at 48 hours fungal incubation was similar to 24 (data not presented). A dark orange pigment characteristic of phenazine compounds appeared at all concentrations of Fe^{+3} and P starting at 24 hours bacterial incubation. The pH of the agar medium directly under the filter membrane was 6.5 to 7.0 following each incubation period, ruling out a direct effect of pH on fungal inhibition.

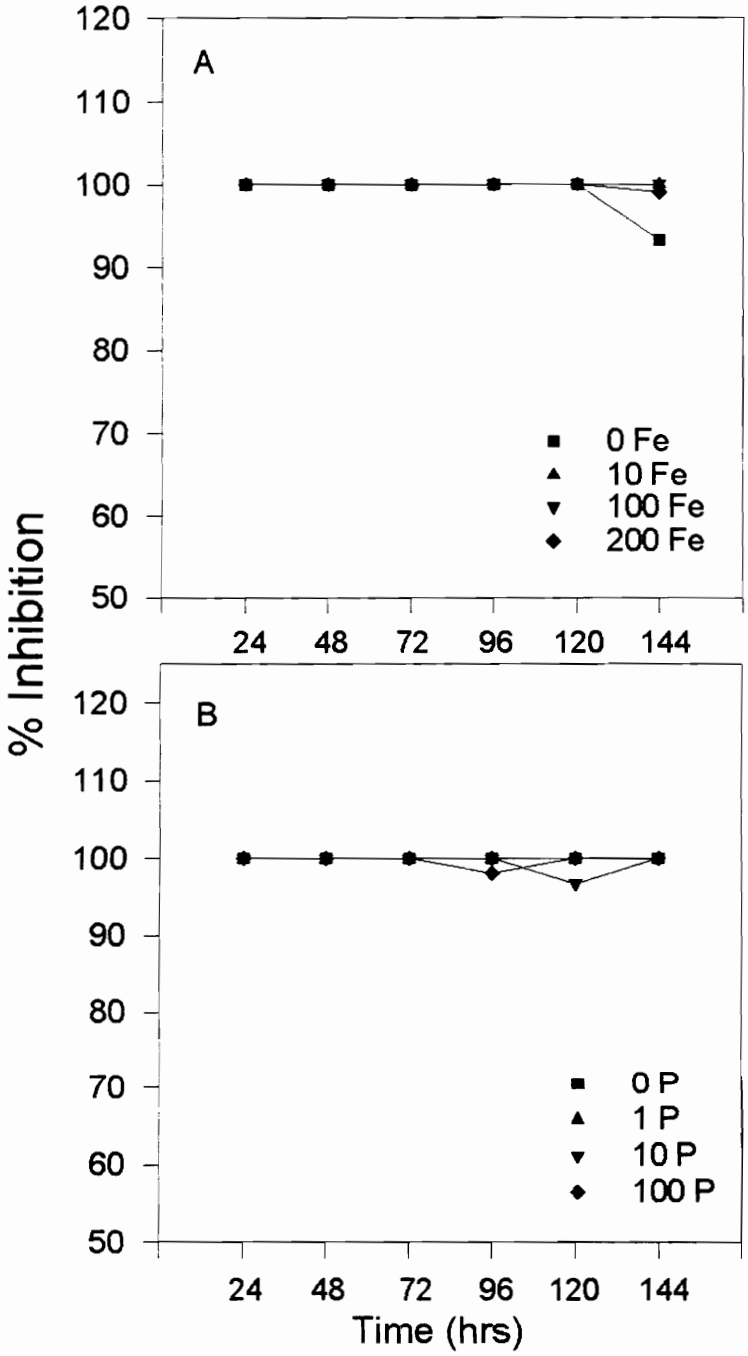


Figure 16. Inhibition of *Pythium ultimum* radial growth (% inhibition relative to nontreated control) by L-850 (wt) antifungal compounds in response to iron (A) and phosphorus (B) concentrations. Time (hrs) is incubation period of L-850 on agar prior to introduction of *P. ultimum*. Growth of *P. ultimum* was measured following 24 hrs incubation.

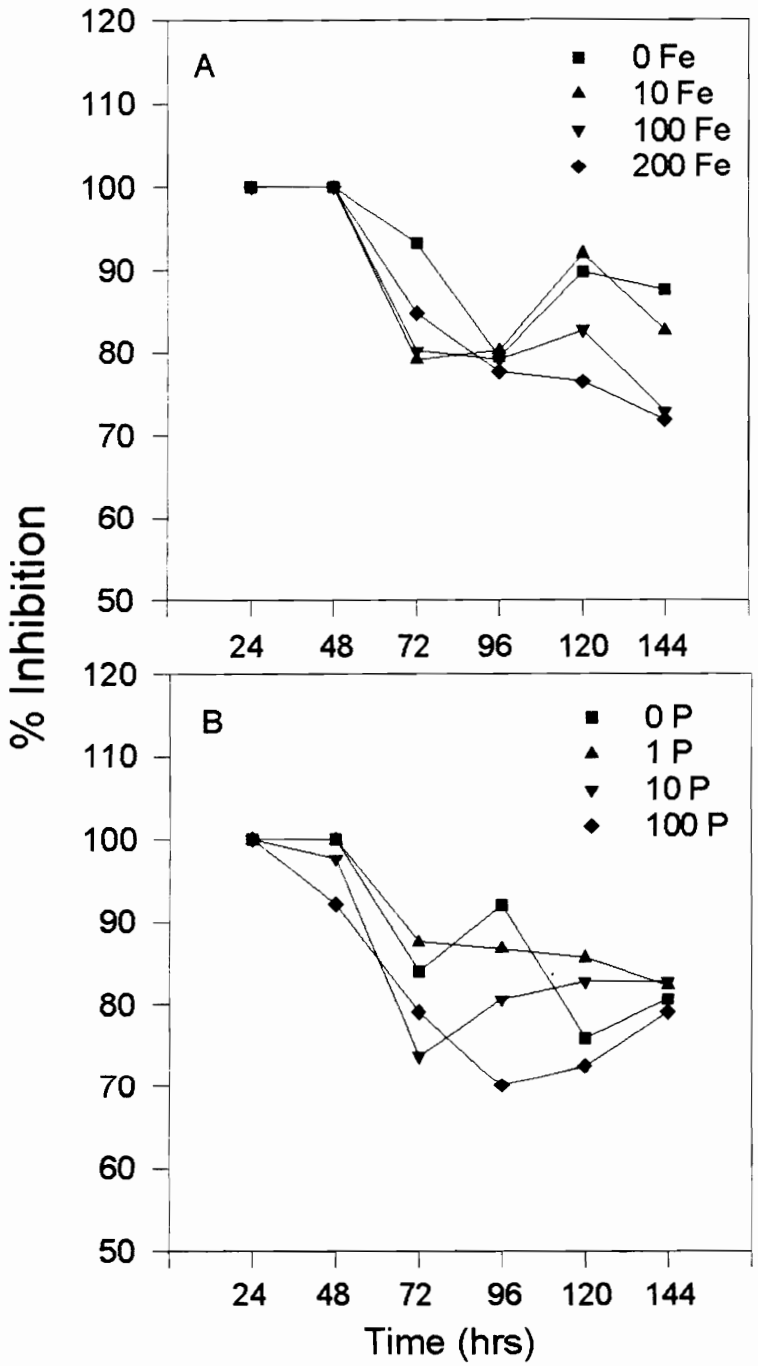


Figure 17. Inhibition of *Rhizoctonia solani* radial growth (% inhibition relative to nontreated control) by L-850 (wt) antifungal compounds in response to iron (A) and phosphorus (B) concentrations. Time (hrs) is incubation period of L-850 on agar prior to introduction of *R. solani*. Growth of *R. solani* was measured following 24 hrs incubation.

APPENDIX C

Non-Filter Paper Agar Diffusion Bioassay - Time Course Study of Antifungal Metabolite Production

Purpose: To study the effect of iron (Fe^{+3}) and phosphorus (P) concentrations on antifungal compounds of *P. chlororaphis* L-850, 1) released upon synthesis from living cells, and 2) from lysed cells (chloroform exposure), over time (6-144 hours incubation).

Protocols:

1. Bacterial inoculum preparation.

Strain L-850 was grown overnight in 500 ml mRSM supplemented with chelated iron in the form of diethylenetriamine pentaacetic acid (DTPA), at 120 rpm and 28 C until a turbidity of 1.03 (A_{640}) was reached. Cells were pelleted, washed twice with 10 mM $MgSO_4$, and resuspended in distilled water.

2. Media preparation.

Modified RSM agar medium (pH 7.0) was ammended with 0 to 200 µM Fe^{+3} (supplied as DTPA) and 0 to 100 mM phosphorus (supplied as KH_2PO_4, pH 7.0). Phosphorus concentration was 1 mM P for the iron assay and 100 µM Fe^{+3} for the P assay. Aliquots of P and Fe stock solutions were added to mRSM after autoclaving with 20 ml of agar medium added per 100 mm glass petri dish. Potato dextrose agar (PDA; Difco, Detroit MI) was prepared according to manufacturer's instructions.

3. Fungal inoculum.

PDA plates were inoculated with a single plug (4 mm) of each test fungus and incubated for 24 hours at 25 C.

4. Agar diffusion assay.

A 1 ml suspension of bacterial culture was spread directly onto the surface of the agar and plates dried for 15 minutes in a laminar flow hood. Plates were incubated at 28 C for 6, 12, 24, 48, 72, 96, 120 and 144 hours to allow diffusion of antifungal substances into the agar. Each plate was inverted over its respective lid which contained 30 ml chloroform and plates sealed in ziplock freezer bags. Cells were lysed after 50 minutes exposure to chloroform. Spent bacterial cells were scraped from the agar surface with a spatula using aseptic technique prior to challenge inoculation with a 4 mm plug from the perimeter of a 24 hour *P. ultimum* or *R. solani* culture. A control treatment consisted of 1 ml distilled water prior to fungal introduction.

5. Data collection.

Fungal mycelial growth (mm) was recorded daily for each treatment concentration until control growth reached the perimeter of the petri dish (72 hrs). Inhibition of fungal growth was calculated relative to control growth values. Each time-treatment concentration was replicated twice per test fungus and the experiment repeated once.

Results:

a. *Pythium ultimum* - Inhibition of *P. ultimum* was 100% at all concentrations of Fe^{+3} and P after 6, 12 and 24 hours bacterial incubation measured after 24 hours fungal incubation on the agar plates (Fig. 18). Percent inhibition varied after 24 hours, the increases at 72 (Fe), 96 (P) 120 (Fe) and 144 (P) experienced following a decline in antifungal activity were most likely due to delays in synthesis and excretion of multiple antifungal compounds. The compounds produced after 12 and 24 hours bacterial incubation on agar were fungicidal as no growth occurred when fungal plugs were transferred to fresh PDA plates. Incubation periods of 6, 48, 72, 96, 120, and 144 hours resulted in a fungistatic effect on *P. ultimum* mycelial growth.

b. *Rhizoctonia solani* - Inhibition of *R. solani* varied in response to time and Fe and P concentrations (Fig 19) as was observed with *P. ultimum*. The greatest amount of mycelial inhibition (72-100%) was observed at 6, 12 and 24 hours bacterial incubation. Phosphorus and iron concentrations of 0 mM and 100 µM respectively, were most inhibitory. The bacterial metabolites produced were fungicidal at 12 and 24 hours incubation and fungistatic at the remaining time intervals, as with *P. ultimum*.

Fungal growth inhibition measured at 48 and 72 hours incubation was similar to 24 (data not presented). A dark orange pigment characteristic of phenazine production appeared in the agar at all concentrations of Fe^{+3} and P starting at 24 hours bacterial incubation. Agar pigmentation was pinkish-orange at 12 hours

incubation and colorless at 6 hours. The pH of the medium was measured after each incubation period and consistently ranged from 6.5 to 7.0, ruling out a direct effect of pH on fungal inhibition.

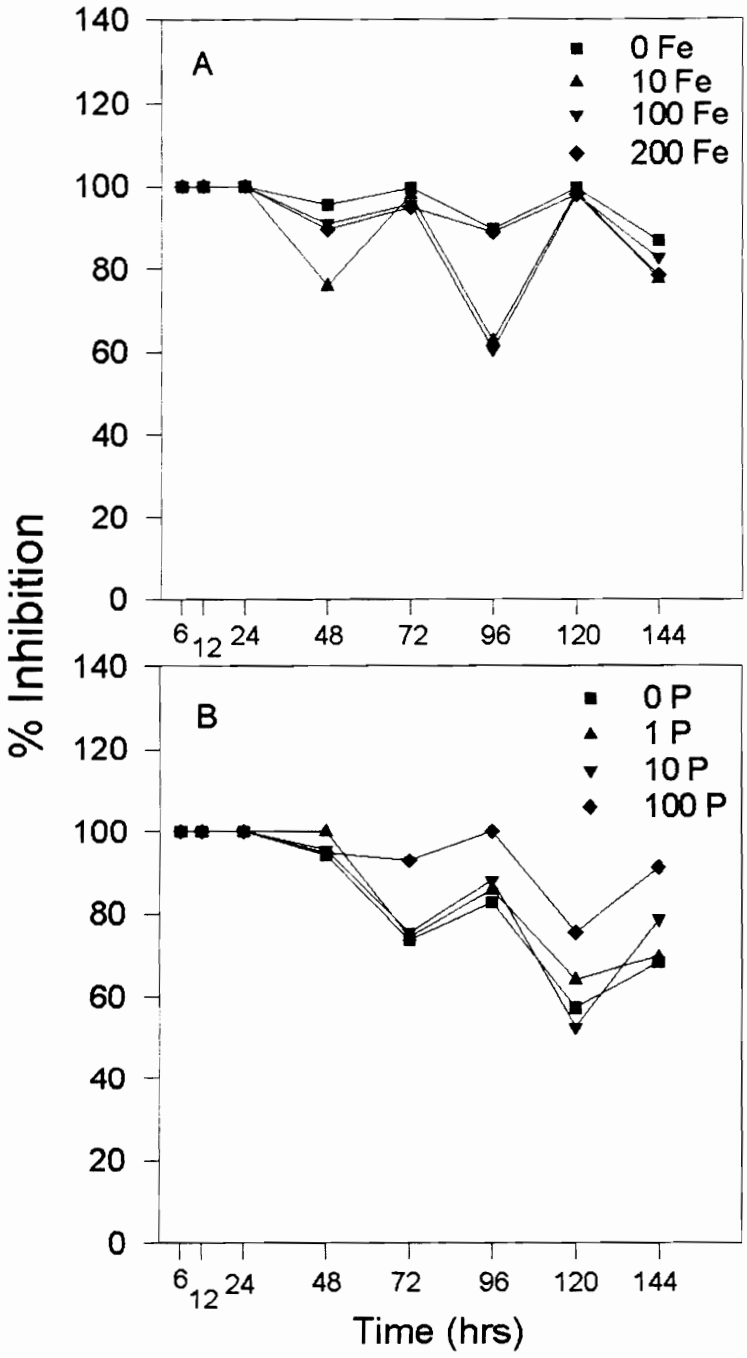


Figure 18. Inhibition of *Pythium ultimum* radial growth (% inhibition relative to nontreated control) by L-850 (wt) antifungal compounds in response to iron (A) and phosphorus (B) concentrations. Time (hrs) is incubation period of L-850 on agar prior to introduction of *P. ultimum*. Growth of *P. ultimum* was measured following 24 hrs incubation.

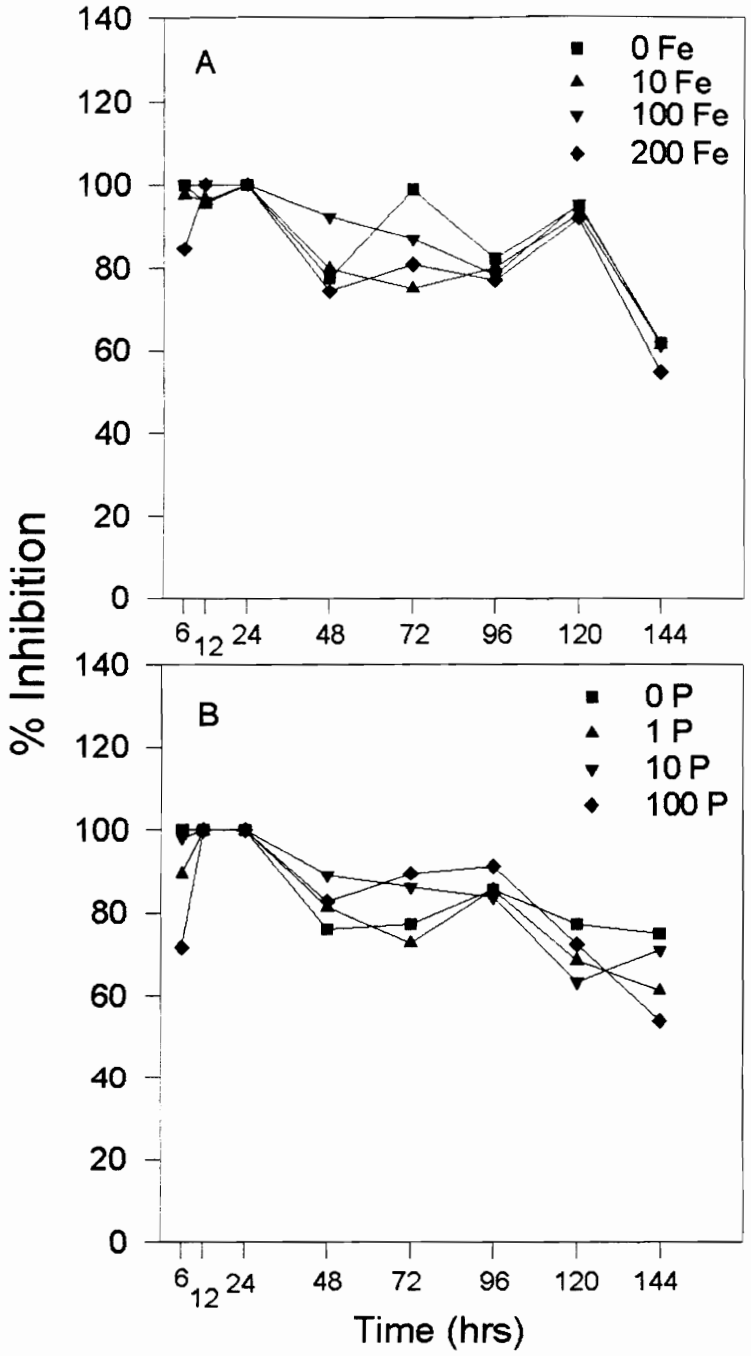


Figure 19. Inhibition of *Rhizoctonia solani* radial growth (% inhibition relative to nontreated control) by L-850 (wt) antifungal compounds in response to iron (A) and phosphorus (B) concentrations. Time (hrs) is incubation period of L-850 on agar prior to introduction of *R. solani*. Growth of *R. solani* was measured following 24 hrs incubation.

APPENDIX D

Extraction of Antifungal Secondary Metabolites from *P. chlororaphis* Strain L-850

Purpose: To separate and crudely purify antifungal secondary metabolites.

A. Protocols (compiled from 12 to 14 citations on secondary metabolite extraction procedures):

I. Bacterial culture conditions and metabolite extraction.

1. A single colony of *P. chlororaphis* L-850 (from -80 C stock) was grown overnight in mRSM with phosphorus (P) and iron (Fe^{+3}) concentrations of 0 mM and 100 µM respectively (See Appendices B and C), at 120 rpm and 28 C until an O.D. (A_{640}) of 0.9.
2. Cells were centrifuged, washed twice with and resuspended in distilled water. A 400 µl aliquot was placed on a 0.22 µ Supor® 200 47 mm membrane filter (Fisher Scientific; Norcross, GA) in the center of a mRSM agar plate (9 plates/extraction; 60 mm diameter) amended with P and Fe^{+3} concentrations as above.
3. Following 24 hours incubation at 28 C, filters were removed and the agar cut into 4 mm^2 segments. Agar squares were then placed in a 250 ml round bottom flask containing 72 ml 80% acetone. The flask was placed on a rotary shaker (170 rpm) for 1 to 1.5 hours.
4. The suspension was filtered to remove agar chunks and evaporated (45 C rotary evaporator/water bath) to remove the acetone. The remaining aqueous phase (pH 3.9) was extracted twice with an equal volume of chloroform ($CHCl_3$):methanol (MeOH) (2:1) and twice with chloroform. Solvent extractions resulted in three separate phases (organic, aqueous, and an oil emulsion).

5. The three phases were treated as follows:
    a) Organic extractions were pooled, evaporated to dryness (N_2 gas), and the white crystals dissolved in methanol (1 ml) and filtered to remove undissolved particulates.
    b) Aqueous extract was evaporated (rotary evaporation; 45 C) to a final volume of 1 ml.
    c) oily extract was evaporated to dryness (N_2 gas), and the residue dissolved in methanol (1 ml).

II. Bioassay for antifungal activity in organic, aqueous and oily phase extracts.

1. Fungal inoculum of *P. ultimum* and *R. solani* was prepared by inoculating potato dextrose agar (PDA; Difco, Detroit, MI) plates with a single plug (4 mm) of each fungus (from stock cultures). Fungi were incubated for 24 hours at 25 C.
2. A 4 mm plug of mycelium from the perimeter of the fungal colony was placed along one edge of a 60 mm mRSM plate amended with P and Fe as above.
3. A 100 µl aliquot of each extraction phase was placed in a well cut in the agar on the opposite side of the plate.
4. Plates were incubated for 24 to 72 hours with growth towards the well (mm) measured daily. Control treatments consisted of chloroform:methanol (100 µl) and nontreated plates.

III. Results:

Growth of *P. ultimum* and *R. solani* mycelium (mm) towards antifungal extract after 24 hours incubation

Treatment	*P. ultimum*	*R. solani*
Nontreated	60	34
$CHCl_3$:MeOH (alone)	60	34
organic extract	0	1
Aqueous extract	14	15
oily extract	60	60

Growth of *P. ultimum* and *R. solani* mycelium (mm) towards antifungal extract after 72 hours incubation

Treatment	*P. ultimum*	*R. solani*
Nontreated	overgrown	52
$CHCl_3$:MeOH (alone)	overgrown	52
organic extract	0	4
Aqueous extract	52	40
oily extract	overgrown	overgrown

IV. Conclusions:

There was no antifungal activity in the oily phase, only partial inhibitor activity in the orange aqueous phase, and highly active compound(s) in the organic phase. *P. ultimum* appeared to be more sensitive to metabolites extracted into the organic phase than *R. solani*. There was no inhibitory effect of the solvent alone. Metabolites that remained in the aqueous phase were more inhibitory to *R. solani* than *P. ultimum*. Extractions and bioassays were repeated 4 to 5 times with similar results.

B. Protocol Modifications (Dr. John Hess, Dept of Biochemistry, VPI & SU):

Modification I. This procedure was essentially as above (Protocol A) with the following changes:

1. The aqueous phase was adjusted to pH 2.0 following evaporation of the acetone to encourage ionized compounds to partition into the aqueous phase.
2. Both the bacteria cells and the agar were extracted with 80% acetone (step 3 above)
3. Use of glass centrifuge tubes (previously polypropylene) to prevent hydrophobic groups from adhering to plastic. All glassware was coated prior to use with Sigmacote® silicone-heptane (Sigma Chemical Co.; St Louis, MO) and dried for 30 minutes at 100 C. Undiluted silanizing material did not inhibit growth of either fungus in *in vitro* tests.

Results: No difference in the inhibitory activity of either the aqueous or organic phase.

Modification II. The procedure was as above (Protocol A) except for the following changes:

1. Add volume of 5% NaCl (1:1 5% NaCl:aqueous phase) following acetone evaporation to reduce oily phase.
2. Extract the aqueous phase three times with $CHCl_3$:MeOH (2:1).

Modification III. As above (A) except:

1. Liquid mRSM with P and Fe concentrations as above, cells grown under shake culture conditions (140 rpm, 24 hrs) followed by 48 hrs stationary culture conditions (non-shake).
2. 100 ml aliquots extracted twice with $CHCl_3$:methylene chloride (the latter to reduce oily/foam phase; Dr. Duane Berry, Dept. of CSES, VPI & SU), and organic phase evaporated (N_2 gas) to final volome of 10 ml.

Results: Zone of inhibition for each fungus increased by 1 to 2 mm.

Modification IV. As above (modification III) except:

1. Cultures incubated under stationary conditions for 144 hours.
2. pH of aqueous phase adjusted to 8.4 with 5N NaOH prior to extraction with $CHCl_3$:methylene chloride.

Results: Zone of inhibition against *R. solani* equivalent to that produced with acidified aqueous phase. The zone of inhibition was reduced by 2 mm for *P. ultimum* relative to that induced by the acidic extract.

APPENDIX E

HPTLC Separation, Elution, and Antifungal Bioassay of Secondary Metabolites from the Organic Extract of *P. chlororaphis* Strain L-850

I. Selection of mobile phase for optimum separation of multiple antifungal compounds (Refs: Handbook of Thin-Layer Chromatography. 1991. J. Sherma and B. Fried (eds), Marcel Dekker, N.Y.; Modern Thin-Layer Chromatography. 1990. N. Grinberg (ed.), Marcel Dekker, N.Y)

Purpose: To separate, purify and characterize antifungal metabolites present in the organic extract (Appendix D, protocol A) using horizontal HPTLC which allows the simultaneous testing of up to 6 mobile phases to identify the mobile phase that produces optimum separation. Compounds identified by HPTLC would be eluted from the adsorbent and tested for antifungal activity by an *in vitro* agar bioassay.

Protocols:

A. HPTLC of organic extract.

1. **Sample preparation (organic extract).** A freezer (-20 C) aliquot of organic extract (in methanol) was evaporated to dryness under N_2 gas and redissolved in acetone (1 ml).
2. **Sample application.** Aliquots (0.5 µl) were repetitively pipetted dropwise, 0.6 cm from the bottom edge of a 10 x 10 cm HPTLC-GHL (hard layer silica gel with inorganic binder; Analtech, Newark, DE) to test a range of concentrations (1-2 µl) of organic extract. Sample zones were dried with a hair dryer (low heat) until complete evaporation of the acetone solvent. Each solvent well contained a different mobile phase. The various mobile phases

tested and the respective sample aliquots loaded were as follows:

Lane 1: Ethyl-acetate (50%); 1 µl

Lane 2: Chloroform:methanol (19:1); 1 µl

Lane 3: Toluene:acetone (4:1); 1.5 µl

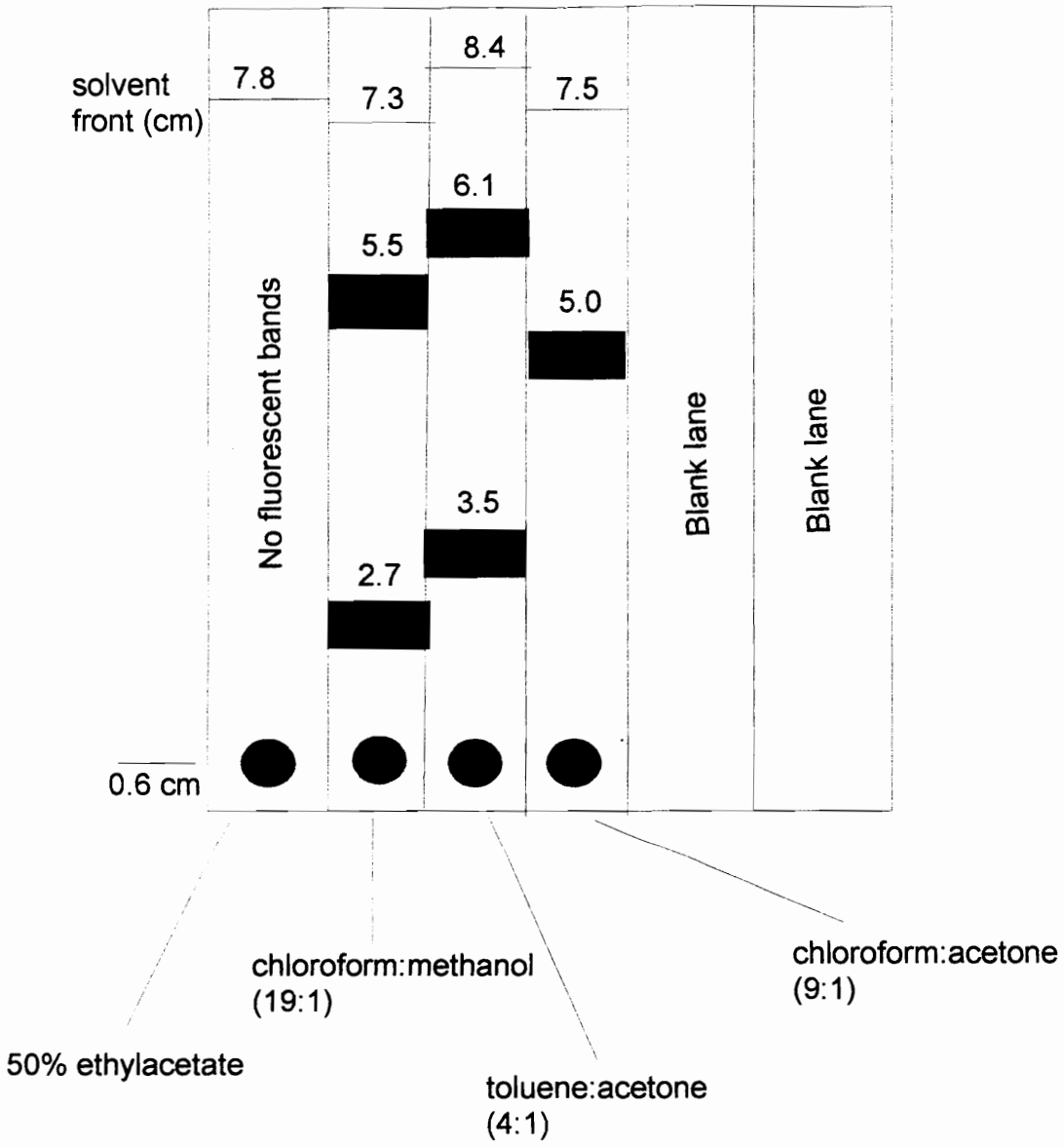
Lane 4 Chloroform:acetone (9:1); 2 µl

The two outer lanes on the right side of the plate were left blank.

3. **Development and visualization of compounds.** The solvents were allowed to migrate a distance of 7 cm across the plate. The plate was then dried at room temperature and exposed to UV_{254} to detect both fluorescent and UV absorbance bands.

The following schematic illustrates the positions of fluorescent bands (cm) in each lane and the solvent migration distance (cm)*.

*Although solvent flow was terminated at a distance of 7 cm, residual migration occured and was taken into account in subsequent R_f calculations.



Migration distance (cm) of fluorescent bands indicated in each lane

R_f values were calculated for the five fluorescent bands.

R_f = distance to center of the band (cm) ÷ distance traveled by the solvent front (cm)

The solvent system and the respective fluorescent compound R_f values in each solvent lane were as follows:

Lane 1: Ethyl-acetate (50%) - no fluorescent compounds detected

Lane 2: Chloroform:methanol (19:1) - R_f = 0.67; R_f = 0.28

Lane 3: Toluene:acetone (4:1) - R_f = 0.65; R_f = 0.34

Lane 4: Chloroform:acetone (9:1) - R_f = 0.58

B. Bioassy for Antifungal activity.

1. Adsorbent partitioning and elution of potential antifungal compounds. Fluorescent bands (above) were numbered 1 to 5. Following removal of the 5 primary adsorbent bands, lanes were subdivided into 1 cm widths each of which were scraped from the plate with a razor blade and placed into 1.5 ml microcentrifuge tubes. A 200 µl aliquot of acetone was added to each tube, and tubes vortexed for 5 minutes to elute the compounds from the silica adsorbent. Tubes were incubated for an additional 5 minutes at room temperature followed by low speed centrifugation for 20 seconds.

2. Agar bioassay (fluorescent/nonfluorescent compound activity). A 4 mm diameter mycelial plug from the perimeter of a 24 hour PDA culture of *P. ultimum* was placed in the center of a 100 mm mRSM-Fe agar plate. The contents of two microfuge tubes were placed opposite one another along the plate edge (two samples/plate), and plates dried in a laminar flow hood until the acetone was evaporated. In addition to the original fluorescent compounds (1-5), each nonfluorescent 1 cm interval adsorbent band was assigned a letter designation (A to R) from top to bottom in each lane as follows:

Lane 1: Ethyl-acetate (50%) - O, P, Q, R;

Lane 2: Chloroform:methanol (19:1) - K, L, M, N; fluorescent bands 4 & 5

Lane 3: Toluene:acetone (4:1) - F, G, H, I, J; fluorescent bands 2 & 3

Lane 4: Chloroform:acetone (9:1) - A, B, C, D, E; fluorescent band 1

Mycelial growth (mm diameter) was recorded after 29 hours incubation at 25 C. Fungal inhibition (%) was calculated relative to growth of mycelium on an acetone only control plate (fungal growth on acetone only = nontreated control growth).

Results:

Band	% inhibition	Band	% inhibition
AB	2.7	MN	6.6
CD	4.0	OP	0
EF	0	QR	8.0
GH	5.3	1,2	5.3
IJ	9.3	3,4,5	5.3
KL	12.0		

Mycelial growth on acetone control plates after 29 hours was 75 mm. The greatest % inhibition was observed with nonfluorescent bands K and L, which corresponded to the top 1 and 2 cm widths closest to the 7 cm solvent front of the chloroform:methanol solvent lane. Loading of a 2 µl aliquot did not produce sharp bands as observed with the loading of 1 µl, indicating sample overloading.

HPTLC in this case was used as a qualitative tool to identify the solvent system for optimum separation of multiple secondary metabolites and to locate compounds with antifungal activity. Hence the low inhibition percentages reflected in the loading of small quantities of crudely purified sample material. A further step would be preparatory TLC plates with a preadsorbent layer designed for the

application of large sample volumes.

APPENDIX F

HPTLC Separation, Elution, and Antifungal Bioassay of Secondary Metabolites from the Organic Extract of *P. chlororaphis* Strain L-850

II. Characterization of structural components of secondary metabolites - chemical reagent visualization following HPTLC (Refs: Ross J.H. 1968. 2,6-dichloroquinone-4-chloroimide as a reagent for amines and aromatic hydrocarbons on thin-layer chromatograms. Anal. Chem. 40:2138-2143.

Purpose: The optimum mobile system identified in Appendix E was used in combination with 2,6-dichloroquinone-4-chloroimide after development, for structure-color reactions on the plate. The reagent detects primary, secondary and tertiary aromatic amines, indoles and other N-containing heterocyclic compounds. The organic extract from strain L-850 was developed along with two known antifungal antibiotics, pyoluteorin and phenazine - 1- carboxylic acid (PCA) for comparison.

Protocols:

A. HPTLC of standards and L-850 extract.

1. **Preparation of HPTLC plate.** The HPTLC-GHL silica gel plate employed previously (Appendix E) was divided into 6 lanes and prewashed with methylene chloride:methanol (1:1). The plate was placed in a developing tank containing 5 ml of prewash solvent for 30 minutes, followed by activation of the plate in a 95 C oven for 30 minutes.
2. **Application of samples and standards.** The following L-850 samples (Appendix D) and standards were applied (from lanes left to right):

   Lane 1: L-850 acid extract

   Lane 2: L-850 acid extract, filtered

   Lane 3: *P. fluorescens* strain 2-79 PCA extract (Strain 2-79 kindly supplied by Dr. David Weller, USDA-ARS; Pullman, WA; PCA extracted according to published protocols: Thomashow, L.S., and Weller, D.M. 1988. Role of

phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. J. Bacteriol. 170:3499-3508.)

Lane 4: pyoluteorin standard (provided courtesy of Dr. C.R. Howell, USDA-ARS; College Station, TX)

Lane 5: L-850 alkaline extract

Lane 6: diphenylalanine (recommended spray reagent standard)

Aliquots were applied as described previously, 2 µl, lane.

3. Development. Development was by vertical ascension utilizing a twin-trough developing tank. The tank was allowed to equilibrate to saturation by filling both troughs with 5 ml of toluene:acetone (4:1). An 8 x 8 cm absorbent pad was placed in one side to facilitate saturation and the tank covered for 1 hour. The plate was then placed in the tank, the top covered as for the equilibration step, and the solvent allowed to migrate a distance of 7 cm. Application of a large sample volume in each lane to facilitate color detection required that the plate be developed a second time using fresh solvent to provide optimum separation.

4. **Visualization (2,6-dichloroquinone-4-chloroimide).** The plate was removed from the solvent tank and dried at room temperature, followed by heat treatment in a 110 C oven for 10 minutes. Upon cooling, the plate was sprayed (0.5 p.s.i.) with a 1.5% solution (in ethanol) of 2,6-dichloroquinone-4-chloroimide until moistened. The plate was then air dried at room temperature and sprayed with a 10% solution of Na_2CO_3. Color reactions were noted.

Results:

I. Response to UV light (254, 366 nm):

Lane 1: L-850 acid extract - 2 fluorescent bands

Lane 2: L-850 acid extract, filtered - fluorescent at application point (no migration)

Lane 3: PCA standard - 1 dark UV absorbing band

Lane 4: pyoluteorin standard - 1 fluorescent band (slight orange tint)

Lane 5: L-850 alkaline extract - 1 fluorescent band

Lane 6: diphenylalanine - no fluorescence

II. Response to 2,6-dichloroquinone-4-chloroimide/Na_2CO_3:

Lane 1: L-850 acid extract - Both bands gray

Lane 2: L-850 acid extract, filtered - no color reaction

Lane 3: PCA standard - light blue band

Lane 4: pyoluteorin standard - purple-violet band

Lane 5: L-850 alkaline extract - no color reaction for fluorescent band

Lane 6: diphenylalanine - dark blue band

Further heating at 110 C (2 minutes) did not result in any additional color reactions.

Conclusion: The unidentified metabolites produced by strain L-850 were not pyrrolnitrin (previous experiments), pyoluteorin, or PCA.

APPENDIX G

Evolution of Hydrogen Cyanide

Purpose: To detect synthesis of the volatile antifungal secondary metabolite hydrogen cyanide (HCN) in *P. chlororaphis* strain L-850.

Protocol:

1. **Bacterial strains.** *P. chlororaphis* strain L-850, a known HCN producing strain, *P. aeruginosa* ATCC 27853, and a negative control *Pseudomonas* spp. strain 1187, were streaked onto a nutrient agar test tube slant and incubated for 24 hours at 28 C.
2. **Detection bioassay.** Strips of Whatman 3 MM filter paper (3 x 0.5 cm) were dipped twice, first in a saturated 0.12% picric acid solution (yellow) followed by a 10% sodium carbonate solution, with drying between solution applications. A single filter paper strip was placed at the top of the test tube taking care to avoid touching the agar surface. Tubes were incubated at 28 C until a color change from yellow to red in the positive control.

Results:

A color change of yellow to red (HCN evolution) occurred for the positive control isolate *P. aeruginosa* but not for either strain 1187 or *P. chlororaphis* L-850.

APPENDIX H

Production of Volatile Antifungal Compounds Other Than HCN

Purpose: To assay for volatile compounds with inhibitory activity to *Pythium ultimum* and *Rhizoctonia solani.*

Protocol:

1. **Fungal inoculum.** Fungal cultures were grown on PDA plates for 24 hours at 25 C.
2. **Bacterial inoculum.** A single colony from a stock culture of *P. chlororaphis* strain L-850 was spread over the surface of a mRSM-FE (100 µM) plate and allowed to incubate for 4 days at 28 C (until a significant lawn of cells formed).
3. **Antifungal bioassay.** A 9 cm glass rod was heated and shaped into a "V". The rod was sterilized and placed on the surface of the bacterial plate. A glass microscope slide on top of which was placed a 2 mm thick slab of fresh PDA was then centered on top of the glass rod. A 4 mm diameter plug of *P. ultimum* or R. solani was removed from the perimeter of a 24 hour culture and placed in the center of the agar slide. Petri dishes were sealed with parafilm, placed inside a ziplock freezer bag in a 25 C incubator and fungal growth monitored at 24 hour intervals for 4 days. Fungal controls were constructed as above but without bacterial application.

Results:

Pythium - Growth from 24 to 96 hours was similar to that of the control plates except that mycelialium did not extend to the bacterial cell surface even after 4 days.

Rhizoctonia - Growth from 24 to 96 hours was similar to the controls, however, after 4 days, hyphal growth had extended from the glass slide and onto the bacterial surface.

Fungal controls - a) *Pythium* - Growth was covering the agar on the glass slide and had extended onto the bacterial surface.

b) *Rhizoctonia* - Growth was covering the agar on the glass slide and had extended onto the bacterial surface.

Conclusions: *P. chlororaphis* may produce volatile compounds with slight activity against *P. ultimum*, but exhibited no effect on *R. solani.*

VIII

VITA

Melinda Anne Mulesky was born and raised in San Francisco, California. In 1977, she obtained an A.S. in Ornamental Horticulture at City College of San Francisco. In 1981 she received a B.S. in Plant Science with a minor in Plant Pathology from the University of California at Davis. After two years of plant pathology experience as a laboratory/field technician in industry, she moved to Blacksburg, Virginia in 1983 to pursue an M.S. in Plant Pathology, and in 1991, a Ph.D. in Plant Physiology in the Department of Plant Pathology, Physiology and Weed Science at VPI & SU. Both the M.S. and Ph.D. research focused on the biological control of soilborne plant pathogens.

Melinda Anne Mulesky