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IDENTIFICATION AND CHARACTERIZATION OF THE SYSTEMIC RESISTANCE-
INDUCING BIOLOGICAL CONTROL AGENT PANTOEA AGGLOMERANS E278A
FROM COMPOST-AMENDED POTTING MIXES.

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of
Philosophy in the Graduate School of The Ohio State University

By

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ABSTRACT

Composts can induce systemic resistance in plants, but this effect is highly variable. This work showed that microorganisms in a compost-amended potting mix were required for the induction of systemic resistance in cucumber (Cucumis sativus L. cv ‘Straight Eight’). This mix contained bacteria that suppress diseases caused by soilborne plant pathogens. Although such bacteria can be excellent biocontrol agents, most were not able to induce systemic resistance in cucumber. However, Pantoea agglomerans strain E278A and the fungus Trichoderma hamatum 382, recovered from compost-amended potting mixes, induced systemic resistance against anthracnose in cucumber and against bacterial leaf spot in radish (Raphanus sativus L. cv. ‘Fuego’). The induced resistance appeared to differ from that mediated by salicylic acid (SA) because PR proteins were not activated in radish or Arabidopsis thaliana (ecotype ‘Col-0’). This suggests that it may be possible to develop inoculants which both induce systemic resistance in plants and control diseases caused by soilborne plant pathogens.

A novel growth pouch bioassay was developed for assessing the ability of microorganisms to induce systemic resistance in radish against bacterial leaf spot. This new bioassay had a number of advantages over earlier bioassays which included miniaturization, greater ease of inoculation, and a shorter test period (14-18 as opposed to 25-30 days). Spatial separation between the biocontrol agent and the pathogen was maintained in this new bioassay and competition from other microorganisms on the plant
was minimized. The new bioassay was also useful for evaluating the efficacy of putative
chemical inducers of systemic resistance. It was used successfully to identify three mini-
Tn5Km-induced mutants of E278Ar that had reduced abilities to induce resistance.

Cell-free protein extracts (CFEs) were made from E278Ar and two non-inducing
mutants by the same method used to prepare harpin from pathogens. These extracts were
as effective in inducing systemic resistance as living cells of wild-type E278Ar, INA, or
harpin_{Es}. This suggested that E278Ar made an elicitor capable of inducing systemic
resistance. CFEs partially lost their ability to induce systemic resistance after treatment
with protease. The activity was heat-stable, indicating that the elicitor may be a harpin-like
protein because harpins also can induce systemic resistance and are also heat-stable. The
CFE from E278Ar induced the hypersensitive response in tobacco, but it did not contain a
protein that cross-reacted with anti-harpin_{Es} antibodies.

E278Ar could possibly be a minor pathogen or have some of the genes required for
pathogenicity because living cells of E278Ar could induce the hypersensitive response in
tobacco. This could also explain its ability to induce systemic resistance, even though it
did not cause symptoms of disease. hrpL is a regulatory gene that is found in all
Pseudomonas and Erwinia/Pantoea hrp clusters. Assuming that this might indicate the
presence of hrp genes, we tested for the presence of hrpL in E278Ar. PCR primers were
designed to hrpL from Erwinia herbicola pv. gypsophila. (Ehg). A weak band
corresponding to hrpL in Ehg was found in E278Ar, and it hybridized weakly to a labeled
hrpL probe. This suggests that some hrp genes may have been present in E278Ar and this,
therefore, might offer an explanation for some of its ability to induce systemic resistance.
DEDICATION

To my grandfathers, Kyung Chik Han and Elwood Houser
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INTRODUCTION

Composts for suppression of plant disease. Composting is the controlled biological oxidation of organic matter by microorganisms (Chen and Inbar, 1993). Mature compost is "stabilized," in that the easily available organic carbon has been oxidized and cellulose, lignin, and humic substances are the major components (Hoitink et al., 1991; Chen and Inbar, 1993). Compost maturity may be determined chemically by measuring the C/N ratio, cation exchange capacity (CEC) or concentration of humic substances (Chen and Inbar, 1993). Biological means for evaluating maturity include respiration rate, rate of general metabolism (for example the rate of hydrolysis of fluorescein diacetate) or bioassays for inhibition of plant growth, a sign of immature compost (Chen and Inbar, 1993; Inbar et al., 1990). The organic matter of composts can also be characterized by NMR or by infrared spectroscopy (Inbar et al., 1989, Inbar et al., 1991; Boehm et al., 1997).

Hoitink et al. (1977) reported the suppression of Phytophthora cinnamomi by composted hardwood bark-amended potting mixes. Mixes amended with other composts also suppress soilborne diseases (Hoitink, 1980; Hoitink and Poole, 1980; Nelson and Hoitink, 1983; Hoitink and Fahy, 1986; Chen et al., 1987; Kuter et al., 1983; Kwok et al., 1987; Craft and Nelson, 1996). Control of root rots caused by soilborne pathogens
with composts can be as effective as that obtained with fungicides (Hardy and Sivasithamparam, 1991; Hoitink et al., 1991). The use of composts in potting mixes for the production of floricultural crops can even replace the use of methyl bromide (Quarles and Grossman, 1995). Compost-water extracts can also be used for control of foliar diseases but their efficacy varies (Weltzein and Ketterer, 1986; Yohalem et al., 1994; Cronin et al., 1996; Zhang et al., 1998). The effectiveness of composts in controlling diseases depends primarily upon the types and activity of the microorganisms living in the compost (Hoitink et al., 1991; Hoitink and Fahy, 1986). In general, more highly decomposed and stabilized composts support fewer species of microorganisms that can act as biological control agents (Hadar et al., 1992; Hoitink et al., 1991), so composts lose the ability to suppress diseases as they age.

Obtaining composts of consistent efficacy is the most important problem in the production of composts for the control of soilborne diseases. Composting is a three-stage process. The initial stage is the metabolism of easily biodegradable substrates and the heating of the compost to about 50° C. Following this stage, thermophilic microorganisms begin to degrade cellulose and the temperature increases to between 60 and 70° C. It is during this peak heating phase that seeds and pathogens in the compost are killed along with most mesophilic microorganisms. For this to occur, compost must be properly turned to expose the entire pile to high temperatures (Bollen, 1993; Farrell, 1993). Because mesophilic organisms are killed during peak heating, the final curing phase is critical for establishing populations of desirable microorganisms in the cured compost. During curing, the metabolic activity of the thermophiles decreases as their food source is depleted and the temperature declines thereafter. This allows recolonization of the compost
by mesophilic microorganisms from the environment. The water content of the compost also has a distinct effect on the final composition of the microflora in the compost, as drier composts tend to be colonized mostly by fungi whereas moist (about 50% water content, w/w) composts tend to have high bacterial populations (Hoitink et al., 1997).

Composts that suppress root diseases contain predominantly Gram-negative bacteria, such as *Pseudomonas* spp. (Kwok et al., 1987; Hardy and Sivasithamparam, 1991). Boehm et al. (1993) found that the relative abundance of these bacteria declined in a peat potting mix as the organic matter decomposed and the mix lost the ability to suppress *Pythium* damping-off of cucumber. Many Gram-negative bacteria that are effective biocontrol agents have been isolated from compost (Boehm et al., 1993). In addition, fungi of the genus *Trichoderma* are sometimes abundant in compost and are also effective biological control agents (Nelson et al., 1983). To promote the recolonization of composts with beneficial microorganisms, they can be inoculated with biocontrol agents immediately after peak heating. For example, inoculating compost with *Trichoderma hamatum* 382 and *Flavobacterium balustinum* 299 results in composts that suppress Rhizoctonia damping-off (Kwok et al., 1987). Compost-amended potting mixes are attractive delivery mechanisms for biological control agents because they provide the proper food and habitat for the biocontrol agent, ensuring its survival. Benefits of composts are not limited to container media, however. They are also effective as topdressings on turf (Nelson and Craft, 1992), as mulches (You and Sivasithamparam, 1994) and as amendments to soil (Alvarez et al., 1995).

Disease suppression induced by composts can involve by four mechanisms: competition, antibiosis, parasitism and predation, and induced resistance. The first two
mechanisms are part of the phenomenon known as "general suppression," where microorganisms create an unfavorable environment for growth of the pathogen, by competing for nutrients, scavenging germination stimulants (van Dijk and Nelson, 1998), or producing antibiotics that inhibit pathogen growth. Predation and parasitism are forms of specific suppression, where one microorganism or a group of microorganisms suppresses a specific target pathogen (Hoitink et al., 1991). This is how diseases caused by *Rhizoctonia solani* and *Sclerotium rolfsii* are controlled by suppressive composts (Nelson et al., 1983; Hadar and Gorodecki, 1990). Recent work in our laboratory (Zhang et al., 1996; Zhang et al., 1998) indicates that composts also suppress disease through the induction of physiological changes in the plant that activate its natural defenses to infection. Activation of systemic resistance by composts is a relatively unstudied phenomenon, yet further research into the mechanisms of activation and the range of diseases against resistance is induced offers the possibility for increasing the use of composts for control of foliar diseases. Induced resistance is an especially attractive mechanism for control of bacterial disease for which no effective control is currently available.

**Concepts and mechanisms of induced systemic resistance.** Plants infected with a necrotizing pathogen often become resistant to subsequent infection by other pathogens. This resistance occurs not only in infected parts of the plant, but in distant, uninfected parts as well. The phenomenon of plants acquiring immunity to disease has been reported in the literature from the beginning of this century. Early studies of the phenomenon reported that inoculation of *Begonia* with less virulent strains of *Botrytis cinerea* made them resistant to infection with highly virulent strains (Ray, 1901). Chester (1933) recounted many examples of induced resistance in a number of host-pathogen systems. More recently, Ross (1961) studied this effect in tobacco using tobacco mosaic
virus and termed it "systemic acquired resistance (SAR). SAR is present in a wide variety of plants, is effective against a broad spectrum of viral, bacterial and fungal pathogens (Delaney, 1997; Ryals et al., 1994; Ryals, et al., 1996), and usually appears in distal parts of the plant several days after the initial infection. The duration of SAR varies according to plant species but generally requires a buildup of salicylic acid (SA) in the plant. (Meera, et al., 1995; Ryals, et al., 1994).

Caruso and Kuc (1977) found that cucumbers, watermelons and muskmelon can be protected from anthracnose in the field by inducing SAR with a small inoculation of Colletotrichum spores. However, SAR can not be induced after the onset of flowering and fruiting (Guedes et al., 1980). Tuzun et al. (1992) reported that inoculation of tobacco with blue mold spores caused SAR in the field against subsequent infection by ambient inoculum. They also noticed that tobacco plants in commercial fields with necrotic stem lesions due to *Peronospora tabacina* had much less severe foliar symptoms than other tobacco plants. This suggests that SAR is activated naturally in these plants. This is assumed to happen frequently (Kuc, 1985), although the extent to which SAR or other induced defenses protect plants in the field is still unknown.

Tobacco plants expressing SAR have altered metabolic activity, particularly phenol metabolism (Simons and Ross, 1971a, Simons and Ross, 1971b). In addition, a novel set of proteins are concomitantly induced in tobacco and other plants undergoing SAR (van Loon and van Kammen, 1970). Van Loon and Antoniuw (1982) named these proteins "pathogenesis related (PR) proteins." PR proteins in plants include peroxidases, chitinases and β-1,3-glucanases (Hammerschmidt et al., 1982; Keller et al., 1996b; Tuzun et al., 1989; Ward et al., 1991). Lamb et al. (1989) distinguished between resistance
genes that are constitutively expressed and those that are activated by pathogen attack or other outside stimuli. Only the latter are involved in SAR. (Ryals et al., 1994).

PR proteins were originally defined as plant-produced proteins that are produced in great amounts only when a plant is infected with a pathogen or under other stress (van Loon, 1983). However, some PR proteins are expressed constitutively at low levels in plants (Memelink et al., 1990). Plant hormones have also been shown to be involved in SAR (Balzas et al., 1977) and, later, induction of PR proteins (Oshashi and Oshima, 1992). Production of PR proteins in various plants can also be induced by stresses such as wounding (Oshashi and Oshima, 1992) and exposure to UV light (Green and Fluhr, 1995). Increases in peroxidase activity were also shown in SAR-induced plants (van Loon, 1976). Lignification plays an important role in SAR along with PR proteins and other plant defense responses (Friend, 1985; Hammerschmidt and Kuc, 1982).

The functions of some PR gene products are known. In tobacco, the PR protein families include β-1,3-glucanases, such as the PR-2 family (Kauffman et al., 1987), which attack fungal cell walls and release glycosides which induce further defense responses in plants (Kombrink et al., 1988; Meins et al., 1992). The PR-3 family is composed of chitinases (Legrand et al., 1987). These also release small chitosan elicitors (Kombrink et al., 1988). The function of the PR-1 family is unknown, but PR-1 has antifungal properties in vitro against Phytophthora (Cutt and Klessig, 1992). Acidic PR-5 proteins are thaumatin-like and may also have antifungal properties (Cornelissen et al., 1986; Linhorst, 1989; Vigers et al., 1991). Basic PR-5 proteins are called osmotins and are involved in salt tolerance in plants (van Loon, 1985).
The identity of the signal molecule that is actually translocated throughout the plant is still unknown, but SA builds up in tissues of induced plants before PR proteins and other defense proteins are expressed (Métraux et al., 1990). Some research suggests that SA is the translocated signal (Malamy, 1990), but more recent work cast doubt upon this (Vemooij et al., 1994). Nevertheless, SA is a vital part of the pathway leading to the establishment of traditional SAR (Malamy et al., 1992; Gaffney et al., 1993; Vemooij et al., 1994) and chemical analogs of SA such as 2,6-dichloroisonicotinic acid (INA) and benzathiadiazoles are able to induce resistance in many plants (Vemooij et al., 1995; Görlach et al., 1996; Kunz et al., 1997). SA may act to regulate hydrogen peroxide levels in the plant by binding to catalase (Chen and Klessig, 1991; Chen et al., 1993), but the importance of hydrogen peroxide in inducing SAR is still in doubt (Hunt et al., 1996; Bi et al., 1995).

Recent research has revealed that accumulation of SA and a buildup of the PR proteins previously described are not absolutely necessary for plants to become resistant (van Wees et al., 1997; Hoffland et al., 1996). Pieterse (1998) has proposed the use of the term "induced systemic resistance" (ISR) to describe cases of resistance that involve the buildup of jasmonic acid (JA) and ethylene rather than SA. JA has previously been shown to act as a signalling molecule between plants (Farmer and Ryan, 1990) and in the response of plants to wounding (Farmer and Ryan, 1992; Creelman et al., 1992) and to affect the activity of phenylalanine ammonia lyase (Gundlach et al., 1992). Melan et al. (1993) showed that a lipoxygenase from Arabidopsis thaliana is inducible by JA, releasing suspected elicitors of defense responses. Mitter et al. (1998) reported the activation of the promoter of an A. thaliana defensin by JA, but not SA. It is expected that more proteins elicited through the ISR pathway will be discovered. ISR offers an alternative mechanism
by which biological control agents can induce systemic resistance without PR protein synthesis, as observed with composts (Zhang et al., 1996; Zhang et al., 1998), some rhizosphere bacteria (Pieterse et al., 1998) and mycorrhizal fungi (Dassi et al., 1998). Clearly, A. thaliana is capable of both SAR and ISR and it is likely that many other plants also employ both responses.

Induction of plant defense responses and systemic resistance by non pathogenic microorganisms. Although the phenomenon of induced systemic resistance was first demonstrated in plants infected by pathogens, non pathogenic microorganisms living on plants or in the soil also induce of defense responses in plants, including SAR and ISR. Systemic resistance can be induced by avirulent or hypovirulent strains of normally pathogenic microorganisms such as Pseudomonas syringae (Cameron et al., 1994), Xanthomonas campestris pv. vesicatoria (Romeiro and Kimura, 1997), Fusarium oxysporum (Martyn et al., 1991; Hervás et al., 1995), or Rhizoctonia (Sneh and Ichielevich-Auster, 1998). It can also be induced by organisms with no known pathogenicity, such as plant growth-promoting rhizobacteria (PGPR) (Wei et al., 1991; Liu et al., 1995a, 1995b, 1995c; Leeman et al., 1995a, 1995b), plant-growth promoting fungi (Meera et al., 1995), or other rhizosphere bacteria and fungi. In many cases, treatment of roots or seeds with biological control agents results in the induction of genes in the SAR pathway, such as PR-1 (Zdor and Anderson, 1992) and other PR proteins (Maurhofer et al., 1994). However, Pieterse et al. (1996) reported systemic resistance can be induced by PGPRs in transgenic Arabidopsis plants containing the bacterial salicylate hydrolase gene nahG. These plants are not able to accumulate SA and are incapable of showing the SAR response, yet they show induced resistance when colonized by PGPRs.
This suggests that many biocontrol agents are able to activate either the ISR pathway in or other, as yet unknown, signalling pathways leading to increased resistance.

Induction of systemic resistance by biocontrol agents has been shown to be dose dependent (Raaijmakers et al., 1995) and can be affected by environmental conditions, most notably iron availability (Leeman et al., 1996; Press et al., 1997). Rhizosphere bacteria induce resistance in plants via a number of different mechanisms. Siderophore production is necessary for induction of systemic resistance by Serratia marcescens strain 90-166 (Press et al., 1998). Some biocontrol agents produce SA (De Meyer and Høfte, 1997). Romeiro and Kimura (1997) reported that purified cell components from incompatible isolates of Xanthomonas campestris pv. vesicatoria induce resistance in pepper. Leeman et al. (1995b) reported that purified lipopolysaccharide (LPS) from Pseudomonas fluorescens induces resistance in radish, and that mutations in the O-antigen can abolish this effect. With fungi, mycelial extracts from Trichoderma longibrachiatum induce resistance and PR proteins in tobacco (Chang et al., 1997). It is likely that as more biocontrol agents that can induce defense responses in plants are discovered, more mechanisms by which they induce systemic resistance will be discovered.

The use of composts as carriers for resistance-inducing biocontrol agents should offer the same advantages as its use as a substrate for other biocontrol agents. Unlike other methods used to apply biocontrol agents, such as seed treatment or sprays, using composts places the desired bacteria directly in the rhizosphere for extended periods of time. This is important because the resistance induced by SAR or ISR does not always last for the life of the crop (Ryals et al., 1994). The challenges in producing composts that consistently are able to induce systemic resistance in plants are the same as those in producing composts.
that suppress pathogens. It is necessary to identify which microbes in the compost are able to induce resistance and under what conditions they thrive. Once these are known, selected resistance inducers can be added to composts just as good pathogen suppressors are added today. The ideal microbe to add to composts would be one which is both a good suppressor of soilborne pathogens and an inducer of systemic resistance. This will potentially increase the value of composts being currently marketed and allow for biological control of more disease than is currently possible.

In order to study systemic induced resistance, care must be taken to distinguish between systemic effects induced in the plant and direct antagonism. This is especially true when working with composts, since the pathogen and biocontrol agent are often present in the soil together. Even in cases of foliar pathogens, bacteria and fungi from the soil often move onto the foliage of plants, especially when water films form on stems and leaves. For this reason, a system which allows spatial separation between the pathogen and putative inducer is highly desirable. Such a system would ideally be soilless and involve only the biocontrol agent of interest. In addition, an assay that takes a short time and is miniaturized is also desirable. Prior to this study, no single assay met all of the above conditions.

The objectives of this research were:

1. To determine whether the previously observed induction of systemic resistance by compost-amended potting mixes is biological in nature, and what kinds of biological control agents are able to induce resistance in plants.
2. To develop a rapid, miniaturized, soil-free assay which allows for screening of putative inducers of resistance and their study.

3. To characterize the mechanisms of resistance induction by biocontrol agent(s) isolated from compost.
CHAPTER 1

SYSTEMIC RESISTANCE AGAINST ANTHRACNOSE IN CUCUMBER
INDUCED BY COMPOST-AMENDED POTTING MIXES AND
INDIVIDUAL BIOLOGICAL CONTROL BACTERIA ISOLATED FROM
COMPOST

Introduction

Composts have long been used to suppress disease in plants. They cause this effect through several mechanisms, including competition, antibiosis and parasitism (Cook and Baker, 1983; Boehm et al., 1993; Hoitink et al., 1997). Several recent reports suggest that composts may also induce systemic resistance to disease in plants. Tränkner (1992) observed that powdery mildew of wheat and barley was less severe on plants grown in compost-amended than control soils. Roe et al. (1993) reported lower incidence of early blight and bacterial spot of tomato on field-grown plants in compost-amended soil than in the control even though the compost was applied under polyethylene mulch. Furthermore, Zhang et al. (1996) showed that composted spruce bark and pine bark-amended mixes induce systemic resistance in cucumber against Pythium root rot and anthracnose caused by Colletotrichum orbiculare.
Systemic resistance can be induced by chemicals, pathogens and beneficial soil microorganisms (De Meyer and Höfte, 1997; Leeman et al., 1995; McQuilken et al., 1994; Mandeel and Baker, 1991; Meera et al., 1995; Pieterse et al., 1996). It is not known how composts induce systemic resistance in plants. However, plant roots grown in compost-amended potting mixes are colonized by a wide variety of bacteria (Boehm et al., 1993; De Brito, et al., 1995; Workneh and van Bruggen, 1994) in taxa from which strains capable of inducing systemic resistance in plants have been described (Liu et al., 1995; Maurhofer et al., 1994; van Peer et al., 1991; Wei et al., 1991). Such specific strains must be present above a certain threshold population level in the rhizosphere to induce this effect (Raaijmakers et al., 1995). Highly decomposed peat mixes that are conducive to root rots caused by *Pythium* and *Phytophthora* spp. do not support high populations of such bacterial taxa (Boehm et al., 1993; Hoitink et al., 1996; Mandelbaum and Hadar, 1990) and do not induce systemic resistance (Zhang et al., 1996). This has led to the conclusion that the potential for substrates to support the activity of biocontrol agents is determined by the microbial carrying capacity of the mix (Boehm et al., 1993). However, recent work in Hoitink’s laboratory (Krause et al., 1998) indicates that even though many compost-amended potting mixes may harbor biocontrol agents capable of providing biological control of diseases caused by soilborne fungi such as *Pythium* and *Phytophthora* spp., very few mixes actually induce systemic resistance under greenhouse conditions.

Some rhizobacteria capable of inducing systemic resistance induce the accumulation of pathogenesis-related (PR) proteins in plants (Maurhofer et al., 1994), whereas others do not (Pieterse et al., 1996; Vidal et al., 1997). This is probably due to the existence in plants of at least two distinct signalling pathways leading to the acquisition of systemic
resistance. The systemic acquired resistance (SAR) pathway (Ryals et al., 1996) involves salicylic acid (SA) as a signal molecule and the accumulation of PR proteins. In contrast, another recently-described pathway, the induced systemic resistance (ISR) pathway (Pieterse et al., 1998) involves jasmonic acid (JA) and ethylene as signals and does not result in the accumulation of SA-inducible PR proteins. Presumably, the accumulation of a different set of proteins is involved in ISR. In fact, one such protein has been identified (Mitter et al., 1998). Pieterse et al. (1998) reported that the rhizosphere bacterium *P. fluorescens* strain WCS417r induces systemic resistance through the ISR pathway.

Zhang et al. (1996) reported that peroxidase activity, a putative SAR marker in cucumber (Graham and Graham, 1991; Rasmussen et al., 1995), is significantly higher in plants grown in a compost mix that induces systemic resistance than those in a peat mix that does not. However, growth in compost alone did not induce the accumulation of high concentrations of PR proteins. It was concluded that the interaction of the compost and pathogen infection appeared critical for rapid activation of SAR-associated gene expression in cucumber plants produced in compost mix (61). Compost water extracts, on the other hand, do activate the synthesis of PR proteins (peroxidase, β-1,3-glucanase) in cucumber and *Arabidopsis thaliana* in concentrations similar to those induced by SA (Zhang et al., 1998). This suggests that the mechanisms by which composts and compost water extracts induce systemic resistance differ. It is not known whether composts are also capable of inducing the ISR pathway, but this appears likely.

The objectives of this work were to determine 1) whether compost-induced SAR is biological in nature, 2) the efficacy of biocontrol agents from suppressive composts in
inducing SAR against anthracnose of cucumber caused by *C. orbiculare*, and 3) whether β-1,3-glucanase activity in Arabidopsis is affected by any of these compost treatments.

**Materials and Methods**

**Potting mixes and plant growth.** Composted pine bark mix and spruce bark mix fortified with the biocontrol agents *Trichoderma hamatum* 382 and *Flavobacterium balustinum* 299 (10^5 and 10^6 CFU g\(^{-1}\) dry weight, respectively) to induce suppression of disease caused by *Pythium*, *Rhizoctonia*, and *Fusarium* (Hoitink et al., 1991; Kwok et al., 1987; Trillas-Gay et al., 1986), were received from Earthgro Inc., Glastonbury, CT. They were prepared as described previously (Hoitink et al., 1996). These batches of mixes also induced systemic resistance in cucumber to *Pythium* root rot and to anthracnose (Zhang et al., 1996). The pine bark compost mix was autoclaved for one hour on three consecutive days in two liter autoclavable bags to eliminate suppressiveness (Kwok et al., 1987; Trillas-Gay et al., 1986). To restore suppression, 10% (v/v) of the biocontrol agent-fortified mix was added back to the autoclaved mix.

Sphagnum peat mixes, used as controls, were prepared with peat that rated H₄ or H₂ on the von Post decomposition-scale (Puutsjarvi and Robertson, 1975). The H₄ mix was consistently conducive to many soilborne pathogens (Hoitink et al., 1991) and did not induce SAR against *Pythium* root rot and anthracnose in cucumber (Zhang et al., 1996). All mixes were amended with slow-release fertilizer (Osmocote, 14-14-14 [N-P-K], Grace-Sierra Chemical Co., Milpitas, Calif.), as described previously (Boehm and Hoitink, 1992), to maintain sufficient nutrients for plant growth.
Cucumber anthracnose bioassay. Cucumber seeds (Cucumis sativus L. cv. 'Straight Eight') were germinated in the compost and peat mixes as described previously (Chen et al., 1988). Plant growth conditions and fertilizer treatments were as described earlier (Zhang et al., 1996). An isolate of Colletotrichum orbiculare, obtained from J. Kúc, Department of Plant Pathology, University of Kentucky, Lexington, was used to induce classic SAR in cucumber. Fourteen days after planting of cucumber seeds, a conidial suspension of C. orbiculare (30 10-µl drops, 10^5 conidia ml^{-1} as determined by hemocytometer counts) was infiltrated into the abaxial side of the first leaf. This treatment is referred to as 'classically-induced' according to Kúc and Richmond (1977). Systemic acquired resistance was detected via challenge-inoculation by placing 30 10-µl drops of a conidial suspension (10^4-10^5 conidia ml^{-1}) of C. orbiculare seven days later on the second leaf of the same plants followed by incubation in a moisture chamber for 24 h in the dark (Kúc and Richmond, 1977; Zhang et al., 1996). The severity of anthracnose was rated seven days after challenge-inoculation as described previously (Zhang et al., 1996). Control plants were treated with water. In some experiments, the first true leaf was treated two days before challenge-inoculation with 5 mM SA as an SAR-positive control.

Bacterial biocontrol agents, selected from over 6000 strains isolated from a composted hardwood bark mix for efficacy against several soilborne plant pathogens (Kwok et al., 1987; Trillas-Gay et al., 1986), were tested for the ability to induce SAR in cucumber against anthracnose. Bacteria were grown for 48 h at 25°C on a rotary shaker in 0.1-strength Difco Trypticase Soy Broth (0.1 TSB). Cells were centrifuged at 10,000 x g and resuspended in autoclaved tap water to establish a concentration of 1 x 10^9 CFU ml^{-1}. These suspensions were injected into the hypocotyl of cucumber seedlings, according to the procedure described by Wrather and Elrod (1990), seven days after seeding in the peat.
mix. For this experiment, efficacy was expressed as the percent reduction in disease severity compared to the control.

In a follow-up bioassay, strain E278Ar, a spontaneous rifampicin resistant mutant of E278A, and strain E371 were grown for 24 h in 0.1-strength TSB, washed, resuspended in sterilized tap water as described above and added to the autoclaved compost mix to establish an initial population density of \(10^6\) CFU g\(^{-1}\) of dry weight mix. Colonization of the mix by these strains was as described earlier (Kwok et al., 1987). Each treatment consisted of two liters of potting mix distributed into five 400-ml pots, with one plant per pot. Pots were arranged in a completely randomized design with five replications per treatment.

The ability of each strain in the compost mix to induce systemic resistance in cucumber was tested by rating anthracnose severity on the second leaf seven days after challenge-inoculation by determining the diameter (mm) and number of lesions, as described earlier (Zhang et al., 1996). Disease severity was expressed as mean lesion diameter (mm). Data were subjected to analysis of variance. When a significant \(P < 0.05\) F test was obtained, separation of treatment means was accomplished using Fisher's least significant different (LSD\(_{0.05}\)) test.

**GUS histochemical staining.** Seeds of transgenic Arabidopsis (*Arabidopsis thaliana* ecotype Columbia), containing a chimeric reporter gene made up of an *A. thaliana* PR gene (*BGL2*) promoter and the coding region of β-glucuronidase (GUS) (obtained from K. Davis, Ohio State Biotechnology Center) were germinated in one-inch diameter pots containing the pine bark compost mix, the peat mix, or compost and peat mixes inoculated with biocontrol agents as described above. Expression of the chimeric GUS reporter gene driven by the β-1,3-glucanase gene promoter (*BGL2*) in transgenic
Arabidopsis plants was determined histochemically by staining whole leaves of plants grown in each mix. Five randomly selected leaves per pot (six pots per treatment) of 28-day-old Arabidopsis plants were excised, surface sterilized in 1% sodium hypochlorite solution for 15 min, washed with distilled water four times and stained overnight at 37°C. The staining solution contained 0.5 mg ml⁻¹ of 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) in 0.1 M Na₂HPO₄ (pH 7.0), 10 mM Na₂EDTA, 0.5 mM K₃ferricyanide/ferrocyanide, and 0.06% Triton X-100 (Jefferson et al., 1987). After staining, leaves were cleared in 75% ethanol overnight with two changes of ethanol, then observed for the development of blue color in the leaves.

Results

**Suppression of anthracnose in compost-amended potting mixes.**

Anthracnose severity on the second leaf of cucumber plants was significantly \((P < 0.05)\) reduced in cucumber plants grown in either composted pine bark mix or composted spruce bark mix as compared to peat mixes (Table 1.1; Figure 1.1). Anthracnose severity on plants grown in the compost mixes was not significantly \((P = 0.05)\) different from the ‘classic-induced’ plants (Table 1.1). Anthracnose severity on plants produced in the autoclaved pine bark compost mix was significantly \((P < 0.05)\) more severe than on those produced in the non-autoclaved mix (Table 1.2, column 1). Anthracnose was also consistently reduced on ‘classic induced’ plants and there was no significant difference in disease severity between ‘classic-induced’ plants and plants grown in the autoclaved mix inoculated with 10% natural composted pine bark mix (Table 1.2, column 1).
<table>
<thead>
<tr>
<th>Mix treatment</th>
<th>Mean lesion diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₄ Peat Mix</td>
<td>3.9</td>
</tr>
<tr>
<td>H₂ Peat Mix</td>
<td>3.4</td>
</tr>
<tr>
<td>Pine Bark Compost Mix</td>
<td>2.4</td>
</tr>
<tr>
<td>Spruce Bark Compost Mix</td>
<td>2.5</td>
</tr>
<tr>
<td>Classic-induced SAR Control</td>
<td>1.9</td>
</tr>
<tr>
<td>LSD₀.₀₅</td>
<td>0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cucumbers were planted in 400 ml of potting mix (five replications per treatment, one plant per replication)

<sup>b</sup>Plants were challenge-inoculated by placing 30 10-µl drops of a 10⁴-10⁵ conidia ml⁻¹ suspension of *C. orbiculare* on the second leaf in a moisture chamber and incubating for 24 h in the dark. Lesion diameter was determined seven days thereafter. Values represent the mean of two experiments

<sup>c</sup>Classic-induced plants were inoculated seven days after seeding by infiltrating a conidial suspension of *C. orbiculare* (30 10-µl drops, 10⁵ conidia ml⁻¹) on the abaxial side of first leaf. Resistance was detected via challenge-inoculation as described above.

Table 1.1. Effects of pine bark compost mix, spruce bark compost mix and peat mixes on induction of systemic resistance against anthracnose of cucumber.
<table>
<thead>
<tr>
<th>Mix treatment</th>
<th>Mean lesion diameter (mm)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test 1</td>
</tr>
<tr>
<td>Non-autoclaved mix</td>
<td>2.9</td>
</tr>
<tr>
<td>Autoclaved mix</td>
<td>3.4</td>
</tr>
<tr>
<td>Autoclaved + 10% non-autoclaved mix</td>
<td>2.0</td>
</tr>
<tr>
<td>Autoclaved + <em>Pantoea agglomerans</em> E278A(^b)</td>
<td>--</td>
</tr>
<tr>
<td>Autoclaved + <em>Pseudomonas putida</em> 371(^b)</td>
<td>--</td>
</tr>
<tr>
<td>Classic-induced in autoclaved mix(^c)</td>
<td>1.6</td>
</tr>
<tr>
<td>LSD(_{0.05})</td>
<td>0.5</td>
</tr>
</tbody>
</table>

\(^a\)Lesion diameter was determined seven days after challenge-inoculation (five replications per treatment, one plant per replication) by placing 30 10-μl drops of a 10^4-10^5 conidia ml\(^{-1}\) suspension of *Colletotrichum orbiculare* on the second leaf in a moisture chamber and incubating for 24 h in the dark.

\(^b\)The biocontrol agents *Pantoea agglomerans* E278A and *Pseudomonas putida* 371 were inoculated into the autoclaved compost mix to establish an initial population density of 10^6 CFU g\(^{-1}\) dry weight mix.

\(^c\)Classic-induced plants inoculated 14 days after seeding by infiltrating a conidial suspension of *C. orbiculare* (30 10-μl drops, 10^5 conidia ml\(^{-1}\)) on the abaxial side of first leaf. Resistance was detected via challenge-inoculation as described above.

Table 1.2. Effects of the compost and selected biocontrol agents on induction of SAR against anthracnose of cucumber in autoclaved compost mix.
Inoculation of the autoclaved pine bark compost mix with *P. agglomerans* E278A significantly (*P*=0.05) reduced anthracnose severity (Table 1. column 2) compared to the autoclaved mix. This response was consistent among three experiments. There was no significant difference in disease severity between 'classic-induced' plants and those grown in the autoclaved compost mix inoculated with *P. agglomerans* E278A. The biocontrol agents *P. putida* 371 (Table 1.2. column 2) and *F. balustinum* 299 (data not shown) were not effective.

**Ability of biocontrol agents to induce systemic resistance in cucumber.** Plants injected with *Pantoea agglomerans* strain E278A had significantly (*P*<0.05) reduced severity of anthracnose as compared to control plants injected with autoclaved tap water (60% and 40% less disease than controls in two experiments) (Table 1.3). Classic-induced plants and those sprayed with 5 mM SA also showed a consistent reduction in disease severity. Plants treated with the biocontrol agents E380B (*Pseudomonas fluorescens*), E76 (*Stenotrophomonas maltophilia*), and A499 (*P. fluorescens*) showed a significant reduction in disease severity in one experiment, but not in a second. Plants injected with several other biocontrol agents consistently did not have significantly less severe (*P* <0.05) symptoms than control plants.

**GUS activity.** After 28 days of growth in peat or compost mixes, with or without inoculation with E278Ar, leaves of *A. thaliana* were stained to detect GUS activity in the leaves. This histochemical staining was negative for GUS activity in most leaves tested. In *A. thaliana* plants grown in autoclaved H₄ peat mix and H₄ peat mix inoculated with E278Ar, a slight blue color was observed in one of five leaves stained, whereas no blue staining was observed in the other leaves (Table 1.4).
### Table 1.3. Reduction of the severity of anthracnose on cucumber by biological control agents injected into the hypocotyl of seedlings.

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Percent reduction of disease severity over control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp.1</td>
</tr>
<tr>
<td>SAR induced</td>
<td>75*</td>
</tr>
<tr>
<td>SA spray (5mM)</td>
<td>72*</td>
</tr>
<tr>
<td>E278A (Pantoea agglomerans)</td>
<td>60*</td>
</tr>
<tr>
<td>E371 (Pseudomonas putida)</td>
<td>49</td>
</tr>
<tr>
<td>E299 (Flavobacterium balustinum)</td>
<td>18</td>
</tr>
<tr>
<td>E380B (Pseudomonas fluorescens)</td>
<td>52</td>
</tr>
<tr>
<td>E76 (Stenotrophomonas maltophilia)</td>
<td>54</td>
</tr>
<tr>
<td>E127 (Pantoea cloacae)</td>
<td>49</td>
</tr>
<tr>
<td>E305 (P. putida)</td>
<td>49</td>
</tr>
<tr>
<td>E222 (P. fluorescens)</td>
<td>8</td>
</tr>
<tr>
<td>E267 (P. fluorescens)</td>
<td>(-11)</td>
</tr>
<tr>
<td>E280 (P. stutzeri)</td>
<td>(-46)</td>
</tr>
<tr>
<td>E106 (P. fluorescens)</td>
<td>NA</td>
</tr>
<tr>
<td>E315 (P. putida)</td>
<td>NA</td>
</tr>
<tr>
<td>A195 (Bacillus sp.)</td>
<td>41</td>
</tr>
<tr>
<td>A499 (P. fluorescens)</td>
<td>40</td>
</tr>
<tr>
<td>E278B (P. agglomerans)</td>
<td>42</td>
</tr>
</tbody>
</table>

*Significantly different (P<0.05) from control (uninduced) plants according to Fisher's LSD test.

Hypocotyls of seven-day-old cucumber seedlings were injected with 100 µl of a 1 x 10⁹ CFU ml⁻¹ suspension of the bacterium to be tested. Bacteria were grown for 24 hr at 25°C in 0.1 TSA before suspensions were prepared in autoclaved tap water. For plants treated with salicylic acid, a 5 mM solution was sprayed on the first true leaf. For plants classically induced for SAR, a 1 x 10⁵ conidia ml⁻¹ solution of C. orbicularae was infiltrated into the underside of the first true leaf. Control plants received no treatment.

Seven days after injection of bacteria, plants were challenge-inoculated with a suspension of C. orbicularae (1 x 10⁵ conidia ml⁻¹, 10 µl per spot, 30 spots per leaf) on the upper surface of the second leaf. Plants were held in a mist chamber for 24 hr, then returned to the greenhouse. Disease severity was assessed, five days after challenge, as the percentage of leaf area covered by necrotic lesions. Data were expressed as a percent reduction in disease severity from that observed in the control.

cNot assayed.
<table>
<thead>
<tr>
<th>Mix treatment</th>
<th>GUS stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>H4 peat</td>
<td>-</td>
</tr>
<tr>
<td>Composted pine bark</td>
<td>-</td>
</tr>
<tr>
<td>H4 peat + E278Ar&lt;sup&gt;b&lt;/sup&gt;</td>
<td>- / +&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Composted pine bark + E278Ar&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Autoclaved H4 peat</td>
<td>- / +&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Autoclaved composted pine bark</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Presence or absence of blue stain in leaves (5 per treatment) after overnight staining in β-glucuronidase substrate solution and clearing in 75% ethanol.

<sup>b</sup>The biocontrol agents *Pantoea agglomerans* E278A and *Pseudomonas putida* 371 were inoculated into the autoclaved compost mix to establish an initial population density of 10<sup>6</sup> CFU g<sup>-1</sup> dry weight mix. Control mixes were treated with water.

<sup>c</sup>Faint blue color in one of five leaves stained.

Table 1.4. GUS activity as determined by histochemical staining of 28 day old *A. thaliana* plants grown in composted pine bark or peat mixes fortified with *P. agglomerans* E278Ar.
Figure 1.1. Cucumber plants showing typical symptoms of anthracnose caused by *Colletotrichum orbicularae*.

The plant on the left was grown in a peat mix (H₄ Peat) and the one on the right in composted pine bark-amended mix (CPB). Note the significant reduction in disease severity on the plant grown in CPB.
Discussion

The results of this study confirm the findings of Zhang et al. (1996) that amendment of a potting mix with compost can reduce the severity of anthracnose of cucumbers by inducing systemic resistance (Table 1.1). Furthermore, this work demonstrates that the ability of the compost mix to induce systemic resistance in cucumber against anthracnose was, at least in part, biological in nature. Autoclaving the mix destroyed most of this ability and inoculation of the mix with a small quantity of natural compost mix restored it (Table 1.2). This suggests that microorganisms in the compost were necessary for induction of systemic resistance. As mentioned before, many beneficial bacteria and fungi induce systemic resistance in plants (Wei et al., 1991; Maurhofer et al., 1994; Leeman et al., 1994; Pieterse et al., 1996; Wei et al., 1995; Liu et al., 1995). These resistance-inducing bacteria are closely related to many bacteria that are abundant in composts suppressive to *Pythium* and *Rhizoctonia* diseases (Kwok et al., 1987; Nelson and Hoitink, 1983; Kuter et al., 1983; Boehm et al., 1993; De Brito et al., 1995). This suggests that a broad spectrum of beneficial microorganisms in composts may induce systemic resistance in plants. However, recent observations by Krause et al. (1998) suggest that the vast majority of composts are not able to induce resistance. In addition, the results of the hypocotyl injection screening (Table 1.3) of biocontrol agents that were effective against *Rhizoctonia* damping-off and *Fusarium* wilt indicate that some of the best biocontrol agents against root diseases were not able to induce systemic resistance. For example, *Flavobacterium balustinum* 299, which was developed as part of a binary compost treatment for inoculation of compost (along with *Trichoderma hamatum* 382) to suppress Rhizoctonia damping off (Kwok et al., 1987) and Fusarium wilt of radish (Trillas-Gay, et
al., 1986), failed to induce systemic resistance in cucumber. This is not surprising, since many mechanisms are involved in suppression of root diseases, most of which have little to do with direct effects on the plant itself. For example, *T. hamatum* 382 was chosen because it is an effective parasite of *Rhizoctonia solani* (Nelson and Hoitink, 1983). *F. balustinum* 299 produces an antibiotic against *R. solani*. Learning more about the microorganisms which do induce resistance allows the opportunity for the development of improved inoculants specifically designed to induce systemic resistance. Identification of the most effective systemic resistance-inducing organisms that also control soilborne plant pathogens could lead to novel disease control strategies and allow biological control to be used more widely.

*P. agglomerans* strain E278A was able to consistently induce systemic resistance in the hypocotyl injection assay (Table 1.3). The spontaneous rifampicin resistant mutant E278Ar was also effective when injected into hypocotyls (data not shown), and when inoculated in the compost-amended potting mix (Table 1.2). This strain was chosen for further studies of the mechanisms of systemic resistance induced by compost-amended potting mixes.

Lack of GUS activity in BGL2-GUS *A. thaliana* plants in the compost mix, the peat mix, or mixes inoculated with the best systemic resistance-inducing biocontrol agent, E2787Ar, agrees with other findings of Zhang et al. (1998). They reported that a quantitative GUS assay was required to detect differences between plants grown in compost and those grown in peat. The results of the GUS histochemical stain suggest that the type of resistance induced by E278Ar differs from classic SAR, which results in a significant accumulation of PR proteins (Kessmann et al., 1994; Kuc and Richmond, 1977; Weymann et al., 1995). Sprays of compost extracts also induce a visible blue color
in histochemical staining of BGL2-GUS *A. thaliana* plants (Zhang et al., 1996). A quantitative assay was not performed in this study.

Zhang et al. (1998) determined that the activity of β-1,3-glucanase in cucumber was not affected by the potting mix in which they were grown until after the plants were infected with *C. orbiculare*. Only after infection with the pathogen was there a difference in the activity of β-1,3-glucanase between plants grown in the compost and in the peat mixes. This difference in activity is slight but significant. However, when plants are classically induced (using *C. orbiculare* spores), and then challenge-inoculated, plants grown in compost-amended mix produce much more β-1,3-glucanase than plants grown in a peat mix. These results suggest that the activity of the compost does not directly induce PR proteins, but conditions the plant to respond by making more β-1,3-glucanase when infected with a pathogen.

Pieterse et al. (1998), as mentioned above, reported a novel pathway in plants leading to systemic resistance induced by biocontrol agents. It involves jasmonic acid and ethylene as signals, not SA. This pathway, termed induced systemic resistance or ISR, does not lead to the accumulation of the PR proteins previously described. However, it is likely that the ISR pathway induces its own set of proteins (Mitter et al., 1998). Because growing cucumbers in a compost mix did not induce high levels of PR protein expression, it is likely that some pathway other than classic SAR is activated by our composts and biocontrol agents.

Although the cucumber/anthracnose bioassay was used successfully in this work to screen a relatively small number of known biological control agents, it had major
drawbacks. First, plants must be placed in a mist chamber for 24 hr prior to inoculation with *C. orbicularae*, and for 24 hr thereafter. This allows for the possibility that biocontrol agents will be able to colonize the foliage of plants from soil. Second, the anthracnose bioassay took almost 30 days to complete and the results were often quite variable. Third, the presence of soil or potting mix in the system means that different chemical properties of different mixes may have effects on disease severity. The *A. thaliana* assay (Simon et al., 1992) and *Fusarium* wilt of radish assay (Leeman et al., 1995a) have similar shortcomings. For screening of large numbers of strains, and characterization of the colonization of roots by the biocontrol agent, a more rapid bioassay would have to be developed. Furthermore, a short term bioassay also should allow better analysis of the mechanisms underlying this induced resistance in plants.
CHAPTER 2

DEVELOPMENT OF A RADISH GROWTH POUCH BIOASSAY FOR
STUDY OF THE INDUCTION OF SYSTEMIC RESISTANCE BY E278Ar

Introduction

Several rhizosphere microorganisms have been identified that can induce systemic resistance in plants. Braun (1923) reported that heat-killed cells of *Agrobacterium tumefaciens*, injected into daisy stems, could induce resistance to infection by live cells. Strains of plant growth-promoting rhizobacteria (e.g. *Pseudomonas* and *Serratia* isolates) induce resistance in cucumber to anthracnose (Vemooij et al., 1995) and Fusarium wilt (Liu et al., 1995a), angular leaf spot (Liu et al., 1995b), tobacco necrosis virus (Maurhofer et al., 1998) and cucumber mosaic cucumovirus (Raupach et al., 1996) in the greenhouse and also under field conditions (Wei et al., 1996). Plant growth-promoting fungi isolated from the rhizosphere of zoysiagrass plants induce resistance in cucumber against anthracnose (Meera et al., 1995), and an isolate of *Penicillium oxalicum* has been reported to induce resistance to *Fusarium* wilt of tomato (De Cal et al., 1997). A heat-stable mycelial extract from *Trichoderma longibrachiatum* has been isolated that induces resistance and PR proteins in tobacco (Chang et al., 1997). Non-pathogenic strains of *Rhizoctonia* spp. can induce resistance in cucumber seedlings against pathogenic *R. solani*.
strains or *Pythium* (Sneh and Ichilevich-Auster, 1998). Also, infection of tomato roots with an arbuscular mycorrhizal fungus induced systemic resistance against *Phytophthora parasitica* in a split-root assay (Cordier et al., 1998). The number of rhizosphere-dwelling microorganisms that have been identified as able to induce plant resistance is constantly increasing.

Not all rhizobacteria can induce systemic resistance in plants. Those that do generally have different effects on different plant species and cultivars (Liu et al., 1995c; van Wees et al., 1997). Most of the known rhizobacteria that can induce resistance in plants are pseudomonads. One mechanism by which rhizobacteria induce resistance is by producing SA (De Meyer and Hofte, 1997), but not all rhizobacteria require SA production to induce resistance in plants. Press et al. (1997) demonstrated that although *S. marcescens* strain 90-166 produces SA, mutants unable to make SA still induce resistance. Nevertheless, introduction of genes for SA production into a *P. fluorescens* strain which did not produce SA, but induced resistance against tobacco necrosis virus in tobacco, resulted in a dramatic increase in the strain’s ability to induce systemic resistance (Maurhofer et al., 1998). Pieterse et al. (1998) showed that the resistance induced by *P. fluorescens* WCS417r is ISR, not SAR. Clearly, bacteria can induce several different pathways in plants, all of which lead to the observed phenotype of induced systemic resistance.

Exogenously applied SA can protect plants from infection by a variety of pathogens (Keller et al., 1996; Mitter et al., 1998; Sticher et al., 1997) and induce PR proteins (Alexander et al., 1992; Keller et al., 1996). Chemical analogs of SA, including 2,6-dichloroisonicotinic acid (INA) (Vermooij et al., 1995) and benzothiadiazoles (Görlich et al., 1996; Kunz et al., 1997) can also induce SAR. Various pesticides have also been
implicated as SAR inducers (Kessman et al., 1994). However, SA-dependent SAR is not the only mechanism of systemic resistance in plants. Pieterse et al. (1998) recently reported that there is a novel signalling pathway leading to systemic resistance in plants which does not involve the accumulation of SA or induction of PR genes, but rather involves jasmonic acid and ethylene. They termed this pathway induced systemic resistance (ISR). Certain peroxidase genes from the legume Stylisanthes humilis are inducible by JA and pathogens, but not by SA (Curtis et al., 1997). Mitter et al. (1998) identified a gene coding for a defensin from Arabidopsis thaliana (PDF 1.2) which is inducible by jasmonic acid but not by SA. It seems likely that both systems operate in many plants.

In addition to chemical and single biological control agents, composts fortified with a mixture of biological control agents induce systemic resistance to Pythium root rot and anthracnose in cucumber (Hoitink et al., 1997; Zhang et al., 1996). Efficacy of composts varies from batch to batch. Effective composts induce peroxidase and β-1,3 glucanase activity in cucumber (Zhang et al., 1996; Zhang et al., 1998), but most of the activation does not occur until after the plant is invaded by a pathogen. It was concluded, therefore, that the composts “prime” the plant to better defend itself when infection occurs (Zhang et al., 1998). Pantoea agglomerans E278Ar is a biocontrol agent effective against Pythium and Rhizoctonia and also induces systemic resistance against anthracnose when inoculated into sterilized composts. Other biological control agents effective against root pathogens are not effective inducers of systemic resistance against anthracnose in cucumber. (Zhang et al., 1998).
Studies of induced systemic resistance in cucumber are hampered by the shortcomings of the bioassay. These include a long duration and the need for the use of a mist chamber, which allows for the possibility that biocontrol agents migrate from the soil to the foliage. In addition, the presence of soil in the bioassay means that effects of the chemical and physical properties of the soil may confound the results. In order to draw conclusions about whether an induced response is systemic, absolute spatial separation of the pathogen and the putative inducer is necessary. For this reason, an alternative to the cucumber/anthracnose bioassay was desirable.

Radish has often been used as a system for investigating systemic resistance induced by rhizosphere bacteria. Leeman et al. (1995a) showed that *P. fluorescens* strain WCS374 induces resistance to Fusarium wilt in radish. They also showed that at least one determinant of resistance induction by *P. fluorescens* is the O-antigen of the lipopolysaccharide (Leeman et al., 1995b). Their work also indicated that the effectiveness of resistance induction in radish plants is affected by the levels of iron available in the growth medium (Leeman et al., 1996). Resistance induced by nonpathogenic organisms in radish may not be accompanied by the buildup of SA and PR proteins whereas resistance induced by a necrotizing pathogen is (Hoffland et al., 1995; Hoffland et al., 1996). This is also true for *A. thaliana* (Pieterse et al., 1998; van Wees et al., 1997). Hoffland et al. (1995) also reported that exogenously applied SA can induce resistance in radish, whereas INA cannot. However, since *Fusarium* enters radishes through the roots, and biocontrol agents are also applied to the roots, spatial separation may not always be achieved. An assay utilizing a foliar pathogen, particularly one for which current control methods are ineffective, would provide a better model for evaluating the efficacy of induced systemic resistance.
The objectives of this study were to: 1) develop a rapid laboratory bioassay utilizing radish and a bacterial foliar pathogen for screening of rhizobacteria for the ability to induce systemic resistance; 2) determine whether resistance induced by these rhizobacteria is comparable to resistance induced by chemicals; and 3) use the bioassay to isolate mutants unable to induce resistance. We report here the development of a growth pouch bioassay which takes only 14-18 days from the time of seeding to the time of disease rating. This assay was used to evaluate the efficacy of *P. agglomerans* E278Ar as an inducer of systemic resistance and for isolating bacterial mutants with reduced ability to induce systemic resistance.

**Materials and Methods**

**Bacterial strains:** *P. agglomerans* strain E278A was isolated from a composted hardwood bark potting mix (Kwok et al., 1987). Strain E278Ar, a spontaneous mutant resistant to rifampicin, was isolated by streaking E278Ar onto 0.1 strength TSA (Trypticase soy agar, BBL) with 50 μg ml⁻¹ rifampicin. Other bacterial strains were isolated from the composted hardwood bark potting mix described above (Kwok et al., 1987). Xanthomonas campestris pv. armoraciae strain (Xca) 704 was isolated from radish in Ohio (Sahin and Miller, 1996). Strain 704 is naturally resistant to 100 μg ml⁻¹ streptomycin.

**Growth of radish seedlings:** Radish (*Raphanus sativus* L. cv. ‘Fuego’) seeds were surface sterilized for 10-15 min in 10% Clorox and rinsed five times with autoclaved tap water. Seeds were then planted in paper troughs in growth pouches (Mega
International, St. Paul, MN) containing 10 ml of 0.25 strength modified Hoagland's solution (Hoagland and Amon, 1938), pH 6.6, containing 5 mM Ca(NO₃)₂·4 H₂O; 5 mM KNO₃; 2 mM MgSO₄·7H₂O; 0.1 mM KH₂PO₄; 0.1 mM K₂HPO₄; 0.2 mM MgCl₂·6H₂O; 0.0235 g L⁻¹ Sequestrene 138 (Novartis) and trace elements. Pouches (six seeds per pouch) were placed in holding racks, covered with aluminum foil, then incubated in a growth chamber (Percival, Boone, IA) at 25°C. After incubation in darkness for 24 h, the aluminum foil was removed and seedlings were then incubated under continuous illumination (30-47 µE m⁻² min⁻¹). They were thinned to five plants per pouch three days after germination. Pouches were regularly watered with autoclaved tap water to keep them moist and fertilized once per week with 0.25 strength modified Hoagland’s solution.

**Root treatment with biocontrol agents and chemicals:** *P. agglomerans E278Ar* was grown for 24 h at 30°C in L-broth (Miller, 1972) with rifampicin (50 µg ml⁻¹) on a rotary shaker at 200 RPM. Thereafter, cultures were centrifuged at 3,840 x g for 10 minutes and the supernatant was discarded. Cells were resuspended in autoclaved 0.25 strength modified Hoagland’s solution and 200 µl of a cell suspension of varying concentration was then pipetted directly onto the roots of three-day-old radish seedlings. Five to ten pouches (five seedlings per pouch) were used per treatment. In some experiments, roots were treated with INA (50 µg ml⁻¹ active ingredient in distilled water) or with purified harpin from *Erwinia stewartii* (Harpin₁₅, 0.5 mg ml⁻¹) at a rate of 200 µl per root as positive induced resistance controls. Negative controls were treated with 200 µl per root of sterilized 0.25 strength modified Hoagland’s solution. Pouches were
arranged in a completely randomized design and returned to the Percival incubator for seven days prior to inoculation with Xca.

When other bacteria were used in the bioassay, they were prepared as described above for E278Ar. When the biocontrol fungus *Trichoderma hamatum* 382 was tested using this bioassay, 1 gram of a commercial preparation of *T. hamatum* 382, about $1 \times 10^5$ CFU g$^{-1}$ dry weight, was added directly to pouches and spread out to make contact with the roots of each seedling in the pouch.

**Challenge inoculation with *X. campestris pv. armoraciae* 704b:** Radish seedlings were challenged with Xca seven days after treatment of roots with E278Ar. Inoculum of Xca was grown for 24-48 h at 27°C on a rotary shaker in sucrose-peptone broth (20 g L$^{-1}$ sucrose, 5 g L$^{-1}$ Bacto peptone) amended with streptomycin (50 μg ml$^{-1}$). Cultures were then centrifuged at 3,840 x g for 10 min. For spray inoculation, suspensions ranging from $1 \times 10^6$ CFU ml$^{-1}$ to $1 \times 10^9$ CFU ml$^{-1}$ were prepared in sterile tap water with 0.02% Silwet L-77 (Lehle Seeds, Round Rock, TX) as a wetting agent and then sprayed to runoff onto radish seedlings. Controls were sprayed with 0.02% Silwet L-77 in autoclaved tap water. For dip inoculation, pouches were inverted and radish leaves dipped in suspensions of Xca for one minute. Control plants were dipped in autoclaved water with Silwet. For vacuum infiltration inoculation, pouches were inverted in suspensions of Xca ranging in concentration from $1 \times 10^2$ CFU ml$^{-1}$ to $1 \times 10^6$ CFU ml$^{-1}$, without Silwet, then placed inside a large bell jar. A vacuum of 75 kPa was applied for two minutes, released, then applied two more times until the tissue of the leaves was
visibly watersoaked. After challenge inoculation, plants were returned to the incubator for four to five days to allow development of symptoms.

Severity of bacterial spot on the first true leaves of seedlings was rated four to five days after challenge inoculation using a disease severity scale in which 1 = symptomless; 2 = fewer than three lesions/leaf; 3 = more than three lesions/leaf, less than 25% leaf area affected by disease; 4 = lesions covering 25-50% of the leaf surface area; 5 = lesions covering more than 50% of the leaf surface area and 6 = one or more leaves dead (Figure 2.1). Disease severity data were analyzed by ANOVA and where a significant ($P<0.05$) $F$ value was observed, separation of means was accomplished using Fisher’s LSD method.

**Determination of bacterial populations in planta:** The populations of the biocontrol agent E278Ar on roots were determined by grinding the roots of one seedling per pouch in 3 ml 0.1 M phosphate buffer (pH 7) in a glass tissue homogenizer. This suspension was then plated on L-agar (L-broth with 1.7% Bacto Agar) amended with 50 $\mu$g ml$^{-1}$ rifampicin (four replicates per root) and the populations were expressed as CFU per root. Populations on leaf surfaces were estimated by immersion of detached leaves (one plant per pouch) in 3 ml 0.1 M phosphate buffer (pH 7) on a rotary shaker for three hours. The washate was then diluted and aliquots plated on LB agar with rifampicin. Populations on and in leaves were then determined by weighing and grinding individual leaves in phosphate buffer (3 ml per leaf) followed by dilution plating on L-agar with rifampicin. These two populations were expressed as CFU g$^{-1}$ fresh weight. All population data were log transformed and analyzed by ANOVA. When a significant ($P<0.05$) $F$ value was observed, separation of means was accomplished using Fisher’s LSD method (Fisher, 1949).
The population of \textit{Xca} on and in leaves was determined by weighing and grinding leaves of one plant per pouch (ten plants total) in 3 ml of 0.01M phosphate buffer (pH 7) followed by dilution plating on sucrose-peptone agar with streptomycin (50 \textmu g ml\textsuperscript{-1}, three replicates per plant). All population data were analyzed as described above.

\textbf{Co-cultivation of \textit{P. agglomerans} E278Ar and \textit{X. campestris} pv. \textit{armoraciae} 704:} E278Ar and \textit{Xca} were cross streaked on L-agar, 0.1 strength L-agar. Trypticase soy agar (TSA) (Difco), 0.1 strength TSA, water agar amended with ground radish leaves (5 g L\textsuperscript{-1}), \textit{Erwinia} inducing medium (2 mM ammonium sulfate, 1 mM potassium phosphate, mM magnesium sulfate, 100 mM 2-[N-Morpholino] ethanesulfonic acid (MES), 1% sucrose, 0.1% casamino acids, pH 5.5 and pH 6.5) and M9 minimal agar (Miller, 1972) to determine whether E278Ar inhibited growth of \textit{Xca} on solid media. Also, log-phase cultures of E278Ar and \textit{Xca} were inoculated (one loopful) either separately or together into 50 ml of broth. Experiments were carried out using both L-broth and M9 minimal broth. At intervals during growth, aliquots were removed from these cultures and a triplicate dilution series in 0.1 M phosphate buffer (pH 7) was plated onto sucrose-peptone agar with streptomycin (100 mg ml\textsuperscript{-1}) to determine the population of \textit{Xca}.

\textbf{Mini-Tn5Km Mutagenesis of \textit{P. agglomerans} E278Ar:} Mini-Tn5 Km was introduced into E278Ar using \textit{E. coli} strain SM10 \lambda PIR(pUTmini-Tn5Km) (de Lorenzo, et al., 1990) via an overnight filter mating. Transconjugants were selected on L-agar containing 50 \textmu g ml\textsuperscript{-1} rifampicin and kanamycin. The frequency of transposition was
approximately $10^6$ per recipient. Transconjugants were plated on both L-agar and M9 (minimal) medium. Individual colonies able to grow on both media were picked and stored at -20°C in 25% glycerol. Putative mutants were screened using the growth pouch radish/Xca assay described above (2 x $10^9$ CFU ml$^{-1}$ suspensions of all mutants were inoculated onto roots and 2 x $10^8$ CFU ml$^{-1}$ suspensions of Xca were used for challenge inoculations, five pouches/mutant), and mutants showing a loss of biocontrol ability were tested a total of three times.

Results

Suppression of bacterial leaf spot by 

$P.\ agglomerans$  

E278A: To determine whether root treatment with E278Ar could reduce bacterial spot severity, roots of radish seedlings were treated with suspensions of the biocontrol agent. Typical symptoms developed on the first true leaves of radish seedlings within five days after inoculation with Xca. Mean disease severity on treated seedlings (2 x $10^9$ CFU per root) was 2.2, significantly ($P<0.03$) lower than the mean rating of 3.2 observed on control seedlings (Table 2.1, Figure 2.2). Similar results were obtained in four separate experiments. Untreated, uninoculated seedlings did not show any symptoms. Furthermore, seedlings treated with E278Ar but not inoculated with Xca also remained symptomless on both roots and leaves. Root treatment with E278Ar more effectively reduced bacterial spot severity when plants were inoculated with a $10^8$ CFU ml$^{-1}$ suspension than a $10^6$ CFU ml$^{-1}$ suspension of Xca (Figure 2.3), but in both cases disease was less severe on plants inoculated with E278Ar than on the control plants. When Xca was recovered on sucrose-peptone agar with streptomycin from the foliage of inoculated
Figure 2.1. Bacterial spot disease rating scale used in the radish growth pouch bioassay.

In this assay, 1 = symptomless; 2 = fewer than three lesions/leaf; 3 = more than three lesions/leaf, less than 25% leaf area affected by disease; 4 = lesions covering 25-50% of the leaf surface area; 5 = lesions covering more than 50% of the leaf surface area and 6 = one or more leaves dead.
Figure 2.2. Bacterial leaf spot symptoms on radish seedlings produced in growth pouches and inoculated on the roots with E278Ar.

Plants were inoculated at 3 days of age and sprayed with a $2 \times 10^8$ CFU ml$^{-1}$ suspension of *Xca* seven days post challenge. A: control plants. B: Plants inoculated on the roots with $2 \times 10^9$ CFU per root of the biocontrol agent *P. agglomerans* E278Ar.
Figure 2.3. Effect of challenge inoculum dose of \( Xca \) on biological control of bacterial spot of radish induced by \( P. \) agglomerans E278Ar.

E278Ar was applied to roots as a suspension of \( 1 \times 10^9 \) CFU ml\(^{-1}\) in 0.25 modified Hoagland's solution to roots of three-day-old seedlings (200 \( \mu l \) per root, five replicates of five plants each). Control plants were treated with 0.25 modified Hoagland's solution (100 \( \mu l \) per root). Plants were challenge inoculated with \( Xca \) seven days after root treatment with E278Ar by spraying leaves to runoff using a suspension of either \( 1 \times 10^6 \) CFU ml\(^{-1}\) (circles) or \( 1 \times 10^8 \) CFU ml\(^{-1}\) (triangles) in sterile tap water with 0.02% Silwet L-77 as a wetting agent. Plants were incubated in a growth chamber under continuous light at 25° C. True leaves were rated for disease symptoms five days following challenge inoculation with \( Xca \) using a disease scale in which 1 = symptomless; 2 = fewer than three lesions/leaf; 3 = more than three lesions/leaf, less than 25% leaf area affected by disease; 4 = lesions covering 25-50% of the leaf surface area; 5 = lesions covering more than 50% of the leaf surface area and 6 = one or more leaves dead.
Root Treatmenta  |  Xca  | Mean Disease Severityb  | Populations of Xca c  
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<tr>
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<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>1.0</td>
<td>&lt;1.5</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>3.2</td>
<td>8.5</td>
</tr>
<tr>
<td>E278Ar</td>
<td>+</td>
<td>2.2</td>
<td>7.4</td>
</tr>
<tr>
<td>LSD0.05d</td>
<td></td>
<td>0.5</td>
<td>0.9</td>
</tr>
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</table>

aRoots were treated three days after germination with 200 µl of autoclaved 0.25 strength modified Hoagland's solution (control) or with 200 µl of a 1 x 10^⁹ CFU ml⁻¹ suspension of E278Ar in 0.25 modified Hoagland's solution (E278Ar). Plants were challenge inoculated on the foliage with a 2 x 10^⁸ CFU ml⁻¹ suspension of Xca seven days following treatment with the biocontrol agent.

bValues represent the mean disease severity ratings for five experiments. True leaves (five replicates of five plants each per experiment) were rated for disease symptoms 6-8 days following challenge inoculation with Xca using a disease scale in which 1 = symptomless; 2 = fewer than three lesions/leaf; 3 = more than three lesions/leaf, less than 25% leaf area affected by disease; 4 = lesions covering 25-50% of the leaf surface area; 5 = lesions covering more than 50% of the leaf surface area and 6 = one or more leaves dead.

cPopulations of Xca were determined by grinding the foliage of five plants per treatment in 0.25 modified Hoagland's and dilution plating on sucrose-peptone agar amended with streptomycin (50 µg ml⁻¹). Data shown are means of two experiments. The detection limit for Xca was 30 CFU g⁻¹ fresh weight leaf tissue.

dLeast Significant Difference (P=0.05) according to Fisher's method.

Table 2.1. Suppression of bacterial leaf spot of radish and the population of Xca in leaves after root treatment with P.agglomerans E278Ar.
seedlings, the population of $Xca$ was an order of magnitude lower on plants treated with E278Ar than on control plants (Table 2.1). Neither $Xca$ nor the biocontrol agent E278Ar were recovered from control plants not inoculated with either E278Ar or $Xca$.

**Effect of varying challenge inoculation methods on ability of E278Ar to suppress bacterial leaf spot.** Radish plants were challenge inoculated with $Xca$ by either dipping leaves in a suspension (2 x 10^8 CFU ml^{-1} with 0.02% Silwet L-77) of $Xca$, spraying this same suspension directly on the leaves, or by vacuum infiltration of a suspension of $Xca$ (1 x 10^4 CFU ml^{-1}, no Silwet). When plants were inoculated with a spray of $Xca$, E278Ar significantly reduced the severity of bacterial leaf spot by one rating unit on the disease severity scale, from 4.0 to 3.0 (Table 2.1, Table 2.2, column 1). Also, when leaves of radish seedlings were dipped in a suspension of $Xca$, the plants treated with E278Ar had significantly less severe bacterial spot than controls (Disease rating of 3.4 versus 4.3, Table 2.2, column 2). Vacuum infiltration of a suspension of $Xca$ into the leaves resulted in disease at much lower concentrations of the pathogen than were necessary to cause disease by spray or dip inoculations. Treatment of roots with E278Ar reduced the severity of disease in plants challenged by vacuum infiltration with 1 x 10^4 CFU ml^{-1} suspension of $Xca$ from 4.3 to 3.6 (Table 2.2, column 3). When leaves were infiltrated with a concentration of 1 x 10^3 CFU ml^{-1}, disease severity was reduced from 2.8 to 2.1. This was also significant ($P = 0.02$). However, the leaves of plants infiltrated with a 1 x 10^6 CFU ml^{-1} suspension of $Xca$ were completely destroyed by bacterial spot whether the roots were treated with E278Ar or not. As noted above, root treatment with E278Ar reduced the severity of bacterial spot in spray treatments at $Xca$ concentrations of 1 x 10^6 and 1 x 10^8 CFU ml^{-1}. Sprays with lower concentrations of $Xca$ resulted in too little.
### Table 2.2. Efficacy of E278Ar in suppression of bacterial leaf spot of radish under several inoculation methods.

<table>
<thead>
<tr>
<th>Root Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>X&lt;sub&gt;ca&lt;/sub&gt;</th>
<th>Mean Disease Severity</th>
<th>Challenge Inoculation Method</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Spray&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>4.0</td>
<td>4.3</td>
</tr>
<tr>
<td>E278Ar</td>
<td>+</td>
<td>3.0</td>
<td>3.4</td>
</tr>
<tr>
<td>LSD&lt;sub&gt;0.05&lt;/sub&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.5</td>
<td>0.7</td>
<td>0.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Roots were treated three days after germination with 200 μl of autoclaved 0.25 strength modified Hoagland’s solution (control) or with 200 μl of a 1 x 10<sup>9</sup> CFU ml<sup>-1</sup> suspension of E278Ar in 0.25 modified Hoagland’s solution (E278Ar). Plants were challenge inoculated on the foliage with a 2 x 10<sup>8</sup> CFU ml<sup>-1</sup> suspension of X<sub>ca</sub> seven days following treatment with the biocontrol agent.

<sup>b</sup>Values represent the mean disease severity ratings for two experiments. True leaves (five replicates of five plants each per experiment) were rated for disease symptoms 6-8 days following challenge inoculation via the spray method (2 x 10<sup>8</sup> CFU ml<sup>-1</sup> suspension of X<sub>ca</sub> in 0.02% Silwet L-77 sprayed to runoff on leaves) using a disease scale in which 1 = symptomless; 2 = fewer than three lesions/leaf; 3 = more than three lesions/leaf, less than 25% leaf area affected by disease; 4 = lesions covering 25-50% of the leaf surface area; 5 = lesions covering more than 50% of the leaf surface area and 6 = one or more leaves dead.

<sup>c</sup>Values represent the mean disease severity ratings for two experiments. True leaves (five replicates of five plants each per experiment) were rated for disease symptoms 6-8 days following challenge inoculation via the dip method (plants inverted in a 2 x 10<sup>8</sup> CFU ml<sup>-1</sup> suspension of X<sub>ca</sub> in 0.02% Silwet L-77 for one minute) using the disease scale described above.

<sup>d</sup>Values represent the mean disease severity ratings for two experiments. True leaves (five replicates of five plants each per experiment) were rated for disease symptoms 6-8 days following challenge inoculation via the vacuum infiltration method (1 x 10<sup>4</sup> CFU ml<sup>-1</sup> suspension of X<sub>ca</sub> in 0.02% Silwet L-77) using the disease scale described above.

<sup>e</sup>Least Significant Difference (P=0.05) according to Fisher’s method.
disease to rate effectively, as did vacuum infiltration with Xca suspensions of less than 1 x 10^3 CFU ml\(^{-1}\).

**Colonization of radish roots by E278Ar applied at several different rates.** Populations of E278Ar on roots and foliage of radish plants were followed to determine whether the biocontrol agent could migrate from roots to shoots. E278Ar grew on roots during the first 24 hours after inoculation (Figure 2.4). Thereafter, populations of E278Ar per individual root remained steady. The final populations were about 5 x 10^7 CFU per root when E278Ar was inoculated at an initial concentration of 1 x 10^8 CFU ml\(^{-1}\) and about 2 x 10^5 CFU per root when inoculated at a concentration of 1 x 10^4 CFU ml\(^{-1}\). Migration of E278Ar from the roots to the foliage was minimal. In one experiment, E278Ar was only recovered from the foliage of 33% of plants tested at the time of disease severity assessment. The mean population of E278Ar on and in the foliage of plants from which it was recovered was less than 100 CFU per seedling. In a second experiment, E278Ar was not recovered from the foliage of any of ten plants assayed. E278Ar was not recovered at all from seedlings not inoculated with the biocontrol agent.

**Efficacy of E278Ar as a function of initial inoculum dose:** To determine how many cells of E278Ar were needed for induction of systemic resistance and suppression of bacterial leaf spot, various initial rates of E278Ar were applied to the roots. E278Ar significantly \((P = 0.05)\) reduced the severity of bacterial spot when inoculated onto roots at initial doses from 1 x 10^5 to 1 x 10^9 CFU per root (Table 2.3). The observed mean disease severity over two experiments was 3.4 for control plants, as compared to 2.4 for plants treated with 1 x 10^9 CFU per root, 2.5 for plants treated with 1 x 10^8 CFU per
Figure 2.4 Effect of initial inoculum dose of E278Ar on colonization of radish roots in growth pouches.

Initial inoculum densities were $1 \times 10^4$, $1 \times 10^6$ and $1 \times 10^8$ CFU ml$^{-1}$ in 0.25 modified Hoagland's solution. E278Ar was applied to roots by dripping 200μl of a suspension onto three day old roots. Entire root systems (five plants per treatment) were harvested and ground in a glass tissue homogenizer, then diluted and plated onto L-agar with rifampicin (50μg/ml). Plates were counted after incubation for 24 h at 30°C.
root and 2.6 for plants treated with $1 \times 10^7$ CFU per root. When plants were treated with initial doses of $1 \times 10^6$ and $1 \times 10^5$ CFU of E278Ar per root, no significant suppression of bacterial spot as compared to control plants was observed (mean disease severity of 3.0 and 3.2, respectively) (Table 2.3). Regression analysis of mean disease severity versus initial inoculum dose yielded an equation of: mean disease severity = -0.112(log CFU per root) + 3.492, and an r-squared value of 0.165. These results were consistent among two experiments.

**Efficacy of E278Ar compared to chemical inducers of systemic resistance.** The mean disease severity on plants treated with E278Ar, when applied to roots at a rate of $2 \times 10^8$ CFU per root, was 3.0, as compared to 3.9 in control plants. Disease severity on plants treated with the chemical inducers INA and harpin$_{Es}$, when applied to roots at a rate of 50 µg ml$^{-1}$ and 0.5 mg ml$^{-1}$, respectively, was 2.5 and 2.7 (Table 2.4) These ratings represent the mean of two experiments. The mean disease ratings for the plants treated with E278Ar were not significantly ($P = 0.05$) different from the chemical treatments, but all three treatments were significantly different from the controls. In addition, the log of the mean population of $Xca$ on and in leaves of plants treated with INA was 6.9, compared to an overall log mean population of $Xca$ of 7.4 for plants treated with E278Ar and 8.5 for control plants. The populations on plants treated with INA and with E278Ar were significantly ($P < 0.05$) different from control plants, but not from each other.
<table>
<thead>
<tr>
<th>Initial E278Ar Dose(^a)</th>
<th>Xca</th>
<th>Mean Disease Severity(^b)</th>
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<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>3.4</td>
</tr>
<tr>
<td>1 x 10^9 CFU per root</td>
<td>+</td>
<td>2.4</td>
</tr>
<tr>
<td>1 x 10^8 CFU per root</td>
<td>+</td>
<td>2.5</td>
</tr>
<tr>
<td>1 x 10^7 CFU per root</td>
<td>+</td>
<td>2.6</td>
</tr>
<tr>
<td>1 x 10^6 CFU per root</td>
<td>+</td>
<td>3.0</td>
</tr>
<tr>
<td>1 x 10^5 CFU per root</td>
<td>+</td>
<td>3.2</td>
</tr>
<tr>
<td>LSD(_{0.05})(^c)</td>
<td></td>
<td>0.5</td>
</tr>
</tbody>
</table>

\(^a\)Roots were treated three days after germination with 200 \(\mu\)l of autoclaved 0.25 strength modified Hoagland's solution (control) or with 200 \(\mu\)l of a suspension of E278Ar of sufficient concentration to produce the indicated initial dose of biocontrol agent in 0.25 modified Hoagland's solution. Plants were challenge inoculated on the foliage with a 10^8 CFU ml^-1 suspension of Xca seven days following treatment with the biocontrol agent.

\(^b\)Values represent the mean disease severity ratings for two experiments. True leaves (five replicates of five plants each per experiment) were rated for disease symptoms 6-8 days following challenge inoculation with Xca using a disease scale in which 1 = symptomless; 2 = fewer than three lesions/leaf; 3 = more than three lesions/leaf, less than 25% leaf area affected by disease; 4 = lesions covering 25-50% of the leaf surface area; 5 = lesions covering more than 50% of the leaf surface area and 6 = one or more leaves dead.

\(^c\)Least Significant Difference (\(P=0.05\)) according to Fisher's method.

Table 2.3. Suppression of bacterial leaf spot in radishes inoculated on the roots with varying initial doses of E278Ar.
E278Ar does not inhibit the growth of Xca in *in vitro* experiments. To determine whether E278Ar inhibited growth of Xca, the two bacteria were grown together on agar and in broth. No zone of inhibition of Xca was observed when they were cross-streaked on L-agar, 0.1 strength L-agar, TSA, 0.1 strength TSA, water agar amended with ground radish leaves, *Erwinia* inducing medium or M9 minimal agar. There was no difference between the growth rate of Xca as a pure culture and its growth rate when co-cultivated with E278Ar in L-broth (Figure 2.5). In minimal (M9) medium, Xca did not grow and died after about six hours. Co-cultivation of Xca with E278Ar in minimal broth delayed the death of Xca cells in comparison to Xca grown by itself (Figure 2.5).

**Mutants of E278Ar unable to control Xca.** Out of 672 mini-Tn5 Km insertion mutants tested, three were identified in preliminary screenings as having lost the ability to control bacterial spot in the growth pouch bioassay. Mean disease ratings of two bioassays on plants treated with mutants 3C8, 3E12 and 4C12 were 3.2, 3.2 and 2.9, respectively, as compared to 3.3 on controls treated with modified Hoagland's solution and 2.4 on plants rated with the wild type E278Ar (Table 2.5). Each of these mutants colonized radish roots as well as the wild-type strain when inoculated at a dose of $2 \times 10^8$ CFU per root (Figure 2.6).

**Efficacy of other biocontrol agents in suppressing bacterial leaf spot in the growth pouch assay.** The growth pouch assay was tested with other biocontrol agents, *Janthinobacterium lividum* strain E275 and *Trichoderma hamatum* strain 382. Plants treated with T. *hamatum* had a mean disease severity among three experiments of 2.2, the same as plants treated with E278Ar (Table 2.6). This was significantly different at
Figure 2.5. Effect of co-cultivation with *P. agglomerans* E278Ar on the growth of the bacterial spot pathogen, *Xanthomonas campestris pv. armoraciae*.

*Xca* was inoculated into L-broth (diamonds) or M9 minimal broth (circles) either alone or with E278Ar and grown at 27°C on a rotary shaker. Populations of *Xca* were determined by diluting aliquots (three per culture) in 0.1 M phosphate buffer and plating on sucrose-peptone agar with streptomycin (50 μg/ml). Plates were counted after 48 hours of incubation at 27°C.
<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Xca</th>
<th>Mean Disease Severity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>3.9</td>
</tr>
<tr>
<td>E278Ar</td>
<td>+</td>
<td>3.0</td>
</tr>
<tr>
<td>INA</td>
<td>+</td>
<td>2.5</td>
</tr>
<tr>
<td>Harpin&lt;sub&gt;E5&lt;/sub&gt;</td>
<td>+</td>
<td>2.7</td>
</tr>
<tr>
<td>LSD&lt;sub&gt;0.05&lt;/sub&gt;</td>
<td></td>
<td>0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Roots were treated three days after germination with 200 μl of autoclaved 0.25 strength modified Hoagland's solution (control), with 200 μl of a 1 x 10<sup>9</sup> CFU ml<sup>-1</sup> suspension of E278Ar in 0.25 modified Hoagland's solution, with 200 μl of a 50 μg ml<sup>-1</sup> solution of INA or with 200 μl of a 0.5 mg ml<sup>-1</sup> solution of purified harpin from Erwinia stewartii. Plants were challenge inoculated on the foliage with a 10<sup>8</sup> CFU ml<sup>-1</sup> suspension of Xca seven days following treatment with the biocontrol agent.

<sup>b</sup>Values represent the mean disease severity ratings for two experiments. True leaves (five replicates of five plants each per experiment) were rated for disease symptoms 6-8 days following challenge inoculation with Xca using a disease scale in which 1 = symptomless; 2 = fewer than three lesions/leaf; 3 = more than three lesions/leaf, less than 25% leaf area affected by disease; 4 = lesions covering 25-50% of the leaf surface area; 5 = lesions covering more than 50% of the leaf surface area and 6 = one or more leaves dead.

<sup>c</sup>Least Significant Difference (P=0.05) according to Fisher's method.

Table 2.4. Comparison of the efficacy of E278Ar for suppression of bacterial leaf spot with the chemical systemic resistance inducers harpin and INA.
<table>
<thead>
<tr>
<th>Root Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Xca</th>
<th>Mean Disease Severity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>3.3</td>
</tr>
<tr>
<td>Wild type E278Ar</td>
<td>+</td>
<td>2.4</td>
</tr>
<tr>
<td>Mutant 3C8</td>
<td>+</td>
<td>3.2</td>
</tr>
<tr>
<td>Mutant 3E12</td>
<td>+</td>
<td>3.2</td>
</tr>
<tr>
<td>Mutant 4C12</td>
<td>+</td>
<td>2.9</td>
</tr>
<tr>
<td>LSD&lt;sub&gt;0.05&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Roots were treated three days after germination with 200 μl autoclaved 0.25 strength modified Hoagland’s solution (Control), or with 200 μl of a 10<sup>9</sup> CFU/ml suspension of E278Ar in 0.25 modified Hoagland’s solution. Plants were challenge inoculated with a 10<sup>8</sup> CFU/ml suspension of Xca seven days following treatment with the biocontrol agent.

<sup>b</sup>Values represent the mean disease severity ratings for two experiments. True leaves (five replicates of five plants each per experiment) were rated for disease symptoms 6-8 days following challenge inoculation with Xca using a disease scale in which 1 = symptomless; 2 = fewer than three lesions/leaf; 3 = more than three lesions/leaf, less than 25% leaf area affected by disease; 4 = lesions covering 25-50% of the leaf surface area; 5 = lesions covering more than 50% of the leaf surface area and 6 = one or more leaves dead.

<sup>c</sup>Least Significant Difference (P=0.05) according to Fisher’s method.

Table 2.5. Loss of suppression of bacterial spot of radish in plants treated on the roots with mini-Tn5Km mutants of the biocontrol agent <i>P. agglomerans</i> E278Ar.
<table>
<thead>
<tr>
<th>Root Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Xca</th>
<th>Mean Disease Severity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>3.2</td>
</tr>
<tr>
<td>E278Ar</td>
<td>+</td>
<td>2.2</td>
</tr>
<tr>
<td>E275 (<em>Janthinbacterium lividum</em>)</td>
<td>+</td>
<td>2.8</td>
</tr>
<tr>
<td>Trichoderma hamatum 382</td>
<td>+</td>
<td>2.2</td>
</tr>
<tr>
<td>LSD&lt;sub&gt;0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Roots were treated three days after germination with 200 μl autoclaved 0.25 strength modified Hoagland’s solution (Control), or with 200 μl of a 10<sup>9</sup> CFU/ml suspension of bacteria in 0.25 modified Hoagland’s solution, or with 1 g commercial *T. hamatum* inoculum, approx. 1 x 10<sup>8</sup> CFU / g dry weight. Plants were challenge inoculated with a 10<sup>8</sup> CFU/ml suspension of *Xca* seven days following treatment with the biocontrol agent.

<sup>b</sup>Values represent the mean disease severity ratings for three experiments. True leaves (five replicates of five plants each per experiment) were rated for disease symptoms 6-8 days following challenge inoculation with *Xca* using a disease scale in which 1 = symptomless; 2 = fewer than three lesions/leaf; 3 = more than three lesions/leaf, less than 25% leaf area affected by disease; 4 = lesions covering 25-50% of the leaf surface area; 5 = lesions covering more than 50% of the leaf surface area and 6 = one or more leaves dead.

<sup>c</sup>Least Significant Difference (*P*=0.05) according to Fisher’s method.

Table 2.6. Use of the radish/bacterial leaf spot growth pouch assay to screen other biological control agents for the ability to induce systemic resistance.
Figure 2.6. Colonization of radish roots by wild type *P. agglomerans* E278Ar and three mutants unable to fully induce resistance.

All bacteria were inoculated onto radish roots at a rate of $2 \times 10^8$ CFU per root ($200 \mu l$ of a $10^9$ CFU/ml suspension in 0.25 modified Hoagland’s solution) and entire root systems (five roots per treatment) were ground in a tissue homogenizer, diluted in 0.25 modified Hoagland’s and plated on L-agar plus rifampicin ($50 \mu g ml^{-1}$). Plates were counted after incubation for 24 h at 30° C.
\( p = 0.05 \) from the control mean disease severity of 3.2. E275 treatment reduced the mean disease severity from 3.2 to 2.8 (Table 2.6), but this difference was not statistically significant.

**Discussion**

These results suggest that E278Ar is an effective inducer of systemic resistance in radish as well as in cucumber. E278Ar applied as a root treatment reduced the severity of bacterial spot by one full disease rating unit, from 3.2 to 2.2 (Table 2.1, Figure 2.2, 2.3). The data presented here demonstrate that the reduction in the severity of bacterial spot in the foliage of radish seedlings treated with E278Ar was due to an induced response. The biocontrol agent did not migrate into the foliage of most plants after it was inoculated onto radish roots, and it did not directly inhibit \( Xca \) on any of several solid media or in mixed broth cultures (Figure 2.5). This indicates that direct interactions between the bacteria did not play a role in the observed suppression of bacterial spot. This evidence and the spatial separation between the pathogen and biocontrol agent on the host suggest that systemic induced resistance was indeed the principal mechanism. However, it is possible that E278Ar could produce an antibiotic on host roots that it does not produce in vitro, and that such an antibiotic could be translocatable in the plant and act directly on \( Xca \) without triggering a physiological response from the plant. That possibility was not investigated in this study. It is also possible that some elicitor of defense responses could be produced by E278Ar on the roots, but that this elicitor is translocated through the plant and induces a local, rather than a systemic, defense response in the leaf.
E278Ar was as effective as INA in inducing systemic resistance in radish to bacterial spot (Table 2.4). This may seem to contradict Hoftland et al. (1995), who reported that INA applied to radish roots did not induce systemic resistance against Fusarium wilt although SA did. However, they based this conclusion on observations of disease incidence, not disease severity. None of the bacterial or chemical inducers they used affected disease severity, but SA and *Pseudomonas fluorescens* strain WCS417 did reduce disease incidence. In this study, disease incidence was not affected by any of the treatments whereas disease severity was reduced by root treatment with INA, E278Ar, and harpin^Es^ (Table 2.4). Harpin has been reported to be an inducer of systemic resistance (Strobel et al., 1996; Jin et al., 1997), so it is not surprising that it induced systemic resistance in this assay. The success in using chemical inducers as positive controls for induction of systemic resistance in this work indicates that this bioassay would be suitable for screening of putative chemical, as well as biological, inducers.

Although E278Ar was able to grow and colonize radish roots when applied at an initial dose of as little as $1 \times 10^4$ CFU per root, the initial dose of E278Ar had an effect on the populations of the biocontrol agent on the roots at the time of challenge with *Xca*. The population of E278Ar was more than an order of magnitude greater on roots treated with an initial dose of $1 \times 10^8$ CFU per root than those treated with an initial dose of $1 \times 10^4$ CFU per root (Figure 2.4). This also had an effect on the efficacy of E278Ar in suppression of disease. An initial dose of at least $1 \times 10^7$ CFU per root was required for significant suppression of leaf spot (Table 2.3). Raaijmakers et al. (1995) reported a dose-response relationship in induction of resistance to *Fusarium* wilt of radish by *Pseudomonas* species, and especially noted that there is a certain threshold population size
necessary to induce this effect. A threshold seems to be in effect in this system as well. Regression analysis did not reveal a linear relationship between initial inoculum dose and final disease rating, perhaps since the final populations on roots was determined by the growth of E278Ar, as well as by initial inoculum dose.

Challenge inoculation method did not affect the efficacy of E278Ar to suppress disease severity (Table 2.2). Although the vacuum-infiltration method required a lower concentration of Xca in order avoid completely overwhelming the radishes, E278Ar was able to lower disease severity even when this inoculation method was used. This suggests that E278Ar is not affecting the entry of bacteria into the leaves, but has some effect on the activity of the pathogen even once it has entered the host. Since there was little difference between the spray and dip methods of inoculation, the spray method was chosen for future work as it was the fastest and most convenient method for challenge inoculation.

We previously reported (Chapter 1; Zhang et al., 1998) that E278Ar induces resistance to anthracnose of cucumber in compost-amended potting mixes fortified with this biocontrol agent. Unfortunately, the anthracnose bioassay, like most other assays used in the screening of biocontrol agents, requires almost one month to complete. These assays therefore are difficult to use for large screening trials. On the other hand, the radish growth pouch assay developed in this work offers the advantage of a short assay period (14-18 days) and requires only a small amount of growth chamber space. Even though plants were grown close together, cross contamination of biocontrol agents between pouches was not a problem. E278Ar was not recovered from control pouches, even though such pouches were located next to those treated with E278Ar. The growth pouch can provide a fast and convenient bioassay for screening of large numbers of potential
biological and chemical inducers of systemic resistance. The assay does have some drawbacks, however. The pouches require frequent irrigation and this assay is not suitable for investigating long-term effects because the seedlings become overcrowded after about three weeks.

Using this bioassay, three mini-Tn5 Km mutants of E278Ar that could not induce systemic resistance in radish were successfully identified. These mutants lost the ability to protect radish seedlings from bacterial leaf spot (Table 2.5), but their growth on artificial media was not affected and their ability to colonize and survive on roots was the same as the wild type (Figure 2.6), indicating that the mutations may prove to be reasonably specific to induction of systemic resistance. Specific mutations have been made in biological control agents to test whether certain gene products such as SA (Press et al., 1997) or LPS (Leeman et al., 1995b) are necessary for induction of systemic resistance. As was noted in preliminary screenings (Zhang et al., 1998), the ability to induce systemic resistance seems to be a rare phenomenon among bacterial biocontrol agents. The growth pouch bioassay developed here offers the possibility of conducting a general, systematic search for both new biocontrol agents capable of inducing systemic resistance and genes required for induction of systemic resistance by biocontrol agents. In fact, the growth pouch was used in this work to determine that \textit{T. hamatum} 382 was also able to induce systemic resistance against bacterial leaf spot in radish.

In summary, this work demonstrated that E278Ar, a biological control agent effective against \textit{Rhizoctonia} damping-off of radish and anthracnose of cucumber, can also induce systemic resistance to bacterial leaf spot of radish. E278Ar is an effective colonizer of radish roots and is as effective as INA and harpin in suppressing the severity of bacterial
leaf spot in radish, even though it has no direct antagonistic effect on *Xca in vitro* and it remains spatially separated from the pathogen. There is an initial threshold dose of E278Ar that is required in order for it to be able to induce a significant reduction in the severity of bacterial leaf spot. Although the method of challenge inoculation with the pathogen impacts the concentration of pathogen necessary to observe differences between plants treated with E278Ar and controls, E278Ar is able to suppress disease when *Xca* is applied as a dip, spray or vacuum-infiltrated.

The growth pouch assay developed in this work is convenient for identifying bacteria, fungal and chemical inducers of systemic resistance and was also used to screen mutants of E278Ar for the loss of the ability to induce resistance. It should also prove to be useful in studies of the mechanisms of this induction of systemic resistance by E278Ar and other biocontrol agents.
CHAPTER 3

PARTIAL CHARACTERIZATION OF INDUCTION OF SYSTEMIC RESISTANCE BY *PANTOEA AGGLOMERANS* E278Ar

Introduction

The previous two chapters show that *P. agglomerans* E278Ar is an effective inducer of systemic resistance in cucumber to anthracnose and in radish to bacterial leaf spot. The radish growth pouch bioassay has also proved useful for the detection of systemic resistance induced by bacteria and chemicals. This makes the growth pouch bioassay a good system for investigation of the mechanisms of induction of systemic resistance by E278Ar.

The signals produced by biocontrol agents that trigger the induction of systemic resistance are not well understood. Leeman et al. (1995b) reported that crude and purified preparations of a lipopolysaccharide (LPS) from *P. fluorescens* WCS374 induces resistance in radish to Fusarium wilt and that O-antigen is required for this induction. In addition, Leeman et al. (1996) found that iron availability affect the induction of systemic resistance because low iron levels increase production of pseudobactin, a fluorescent pyoverdine siderophore from WCS374, which in turn induces systemic resistance in
radish. Production of SA by bacteria also is involved in the induction of systemic resistance in some cases (De Meyer and Höfte, 1997) and can enhance induction in others (Maurhofer et al., 1998). However, Press et al. (1997) reported that SA production was not essential for induction of systemic resistance by Serratia marcescens in cucumber and tobacco. Romeiro and Kimura (1997) reported that crude cell component preparations (LPS, EPS, cell envelopes and the capsular glycoprotein) from both compatible and incompatible isolates of Xanthomonas campestris pv. vesicatoria were able to induce resistance against a compatible isolate when the elicitors were infiltrated into pepper leaves. However, only the membrane fraction and living cells induced phytoalexins, leading to the conclusion that some other form of resistance, possibly SAR or ISR, was induced by the other fractions.

Elicitors produced by plant pathogens also induce systemic resistance. For example, Kamoun et al. (1993) reported that the proteinaceous elicitor of HR from Phytophthora spp. induces resistance in radish to Xca, and Strobel et al. (1996) found that harpin from Pseudomonas syringae pv. syringae induces systemic resistance in cucumber to C. orbicularae, TNV and P. syringae pv. lachrymans. Induction of systemic resistance has also been liked to the ability of bacterial pathogens to induce the hypersensitive response. Although E278Ar is not a pathogen of radish, strains of P. agglomerans pathogenic to beach peas have been described (Khetmalas et al., 1996). Therefore, it is possible that some proteins present in E278Ar may also play a role in its induction of systemic resistance. E278Ar may be a minor pathogen of some host other than radish, or an avirulent strain. Alternatively, some of the genes involved in pathogenicity may also affect benign colonization of roots. If so, this could provide a new framework for understanding the induction of resistance in plants by plant-associated bacteria.
The objectives of this work were to determine whether living cells of E278Ar produced elicitors capable of inducing systemic resistance, and if so, to begin to characterize these elicitor(s) and determine how they induce systemic resistance.

Materials and Methods

Bacterial strains. Bacteria used in this study are listed in Table 3.1.

Preparation of cell-free extracts (CFEs) of bacteria: CFEs were produced via the harpin extraction procedure (Wei et al., 1992). Wild type E278Ar, mini-Tn5Km mutants of E278Ar, or E. coli strain HB101 was grown for 24 h at 30°C in L-broth on a rotary shaker at 200 RPM. Cultures were centrifuged as described in Chapter 2 and the supernatant discarded. Cells were resuspended in autoclaved tap water (0.1 g pelleted cells ml⁻¹) and 100 μl of 0.1 M phenylmethylsulfonyl fluoride (PMSF, a protease inhibitor) was added per 10 ml suspension. The cell suspension was then sonicated for 10 min using a Tekmar model Tm-250 sonic disrupter on intensity setting 4, 40% duty cycle. The sonicate was then boiled for 10 min, cooled on ice and centrifuged for 30 min at 17,540 x g. The resulting CFE was filtered through a membrane filter (pore size = 0.2 μm) and stored at -20°C for later use. To treat roots of radish seedlings, CFEs were adjusted to a total protein concentration of 0.5 mg ml⁻¹ as determined by the Biuret method (Gornall et al., 1949) and dripped onto roots of seedlings as described in Chapter 2. For certain experiments, CFEs were treated with protease by adding 100 μl of 1 mg ml⁻¹ Sigma Type IV protease per ml CFE and incubating for at least 1 hr at 37°C. For other
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. agglomerans</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E278A</td>
<td></td>
<td>Kwok et al., 1987</td>
</tr>
<tr>
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<td>Rif&lt;sup&gt;e&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>3C8</td>
<td>E278Ar::mini-Tn5Km</td>
<td>This study</td>
</tr>
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<td>E278Ar::mini-Tn5Km</td>
<td>This study</td>
</tr>
<tr>
<td>4C12</td>
<td>E278Ar::mini-Tn5Km</td>
<td>This study</td>
</tr>
<tr>
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<td>Boyer and Roulland-Dussoix, 1969</td>
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<tr>
<td>DH5α</td>
<td>F⁻ recA φ80 dlacZ ΔM15</td>
<td>BRL</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>hsdS gal (λIts 857 indI Sam7 nin5 lacUV5-T7 gene f)</td>
<td>Studier and Moffat, 1986</td>
</tr>
<tr>
<td><strong>E. stewartii</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC283</td>
<td>SS104 Nal&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Coplin et al., 1981</td>
</tr>
<tr>
<td>DM3020</td>
<td>DC 283 Δhrp</td>
<td>Coplin et al., 1992</td>
</tr>
<tr>
<td>DM782</td>
<td>DC 283 hrpL::apaA3</td>
<td>D. Majerczak</td>
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<tr>
<td><strong>E. herbicola pv. gypsophilae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC557</td>
<td>nal&lt;sup&gt;r&lt;/sup&gt;</td>
<td>I. Baruch</td>
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<td><strong>Plasmids</strong></td>
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<td>D. Majerczak</td>
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<td>pDM2533</td>
<td>hrpL PCR subclone in pBluescript</td>
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</tr>
<tr>
<td>pMA2</td>
<td>1.8 kb HindIII hrp fragment in PBS</td>
<td>M. Ahmad</td>
</tr>
</tbody>
</table>

Table 3.1. Bacterial strains and plasmids used in this study
experiments, CFEs were dialyzed for 48 hr against distilled water (four changes) using 10,000 MW cutoff Spectra/Por dialysis tubing (Spectrum, Houston, TX).

**Western dot blots.** CFEs were adjusted to a total protein concentration of 0.5 mg ml⁻¹ and spotted onto nitrocellulose membrane (Boehringer Mannheim, Mannheim, Germany) at full strength and at dilutions of 1:1, 1:2, 1:5, 1:20 and 1:60. After the membrane air dried, it was floated in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) until wet, submerged, then rinsed. The membrane was blocked in TBST with 1% BSA for 30 min, then incubated in 50 ml primary antibody serum (1:25,000 dilution of anti-harpinEs in TBST) (Ahmad, 1996) for 1 h, then rinsed three times in TBST. The membrane was then incubated in a 1:5,000 dilution of alkaline phosphatase-conjugated anti-IgG (Sigma, St. Louis, MO) for 30 min. The blot was rinsed three times in TBST and then in TBS without Tween 20. Color development was performed by incubating the membrane in 20 ml Western Blue substrate for alkaline phosphatase (Promega, Madison, WI) until the desired color intensity was achieved.

**Northern blots.** Total RNA was extracted from radish leaves seven days after root treatment with E278Ar or INA. Leaf tissue (100 mg) was harvested and immediately flash frozen in liquid nitrogen. Tissue was either processed immediately or stored at -80°C for later extraction of RNA. To extract RNA, frozen tissue was ground into a powder under liquid nitrogen and RNA was extracted using a QIAGEN (Santa Clara, CA) RNA extraction kit. Total RNA was resuspended in RNase-free water and stored at -80°C.

For northern blots, total RNA was electrophoresed on a denaturing gel (1% agarose, 20% formaldehyde in 1X MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1
mM EDTA, pH 7.0) at 5 V cm\(^{-1}\) in a 10 cm horizontal gel apparatus. All solutions were treated with diethylpolycarbonate (DEPC) and glassware baked at 180° C overnight to eliminate RNAse contamination. After electrophoresis, the gel was rinsed twice in 20X SSC (3 M NaCl, 0.3 M sodium citrate) to remove formaldehyde and RNA samples were blotted onto a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany) overnight via upward capillary transfer and then baked at 80° C for three hours to fix the RNA to the membrane.

DNA probes for the PR-1 and GST-1 genes from *Arabidopsis thaliana* were provided by Dr. K. Davis, Biotechnology Center, The Ohio State University. These probes were nonradioactively labeled with digoxigenin-dUTP (DIG) using a Genius kit from Boehringer Mannheim, Mannheim, Germany. Labeled probes were hybridized to the RNA according to the manufacturer's instructions as follows. The membrane was incubated in hybridization solution (5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% Blocking Reagent) for 2 h, then the solution was decanted and fresh solution containing the labeled probe (100 ng ml\(^{-1}\)) was added. After incubation for 16 h at 68° C, the membrane was washed twice with 2X SSC, 0.1% SDS at room temperature and then twice with 0.1X SSC, 0.1% SDS at 68° C. The membrane was then washed once in maleic acid buffer (0.1M maleic acid, 0.15 M NaCl, pH 7.5) and incubated in blocking solution (1% Blocking Reagent in maleic acid buffer) for at least 2 h. After blocking, anti-DIG-alkaline phosphatase conjugate was added (75 mU ml\(^{-1}\)) and the membrane was incubated for 30 min. After antibody binding, the membrane was rinsed in maleic acid buffer and immersed in full strength Promega (St. Louis, MO) substrate for alkaline phosphatase until the bands were the desired intensity. After color developed, membranes were photographed and wrapped in plastic wrap for storage.
Bacterial DNA extraction. Genomic DNA was extracted from overnight cultures grown in L-broth at 30° C. Five ml of culture were centrifuged at 5000 x g, the culture medium was discarded and the cells were resuspended in 450 μl water with 25 μl Proteinase K (20 mg ml⁻¹) (BRL, Gaithersburg, MD) and 20 μl 25% sodium dodecyl sulfate (SDS). Cells were lysed at 55° C for 4 h, then extracted sequentially with equal volumes of buffer-saturated phenol, phenol : chloroform (1:1) and chloroform. After the final extraction, 20 μl 3 M sodium acetate and 350 μl 95% ethanol were added and the samples were incubated at -20° C overnight. After precipitation of DNA, the samples were centrifuged for 10 min at 15,000 x g and DNA was resuspended in 40-50 μl 10 mM Tris - 1 mM EDTA, pH 8.0.

Plasmid DNA was extracted from overnight cultures grown as described below. Cultures were centrifuged (1.5 ml at 5,000 x g) and cells resuspended in 150 μl lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris, 4 mg ml⁻¹ lysozyme) and vortexed. When thoroughly resuspended, 200 μl of freshly prepared 0.2 N NaOH / 1% SDS solution was added, tubes were inverted, and 150 μl 3M potassium acetate was added. The solution was centrifuged for 5 min at 15,000 x g in a benchtop microcentrifuge and the supernatant removed to a clean tube, extracted with an equal volume of 1:1 phenol : chloroform and centrifuged for 2 min at 15,000 x g. The upper phase was removed to a clean tube and the DNA precipitated with 1 ml 95% ethanol at -20° C for at least 30 min. DNA was then recovered by centrifugation for 10 min at 15,000 x g and resuspended in 40-50 μl 10 mM Tris - 1mM EDTA, pH 8.0. Concentration was determined by reading the absorbance of the DNA solution at 280 nm.
**Polymerase chain reaction.** Primers for amplification of the *hrpL* open reading frame were designed from the coding sequence of *hrpL* from *Erwinia herbicola* pv. *gypsophila* (I. Barash, unpublished data): primer DK1 (forward) (5' - CCGAAGACCTGGTACAGATGACC-3') and primer DK2 (reverse) (5' - CGGCGATATC(rrGATAACTGCC-3') (DNAgency, Malvern, PA). Amplifications were performed in 100 μl total volume using 10 ng of template DNA, 10 μM of each dNTP, 20 nM of each template, and 5 U of *Taq* polymerase in 1X PCR buffer (BRL, Gaithersburg, MD). Either 5 mM or 10 mM (final concentration) magnesium chloride was included in the reaction mix. Reactions were performed for 35 cycles as follows: 95° C for 2 min for initial denaturation, then 40 s at 95° C, 1 min at either 56° C (low stringency) or 58° C (high stringency) and 1.5 min at 72° C. After 35 cycles, a final extension step of 5 min at 72° C was performed.

Amplified DNA was extracted with an equal volume of phenol : chloroform (1:1), then with an equal volume of chloroform. DNA was precipitated as described above, resuspended in 15 μl water, and electrophoresed on 1% agarose (BRL, Grand Island, NY) gels in TAE buffer (40mM Tris, 2 mM EDTA, pH 8.0) at 10 V cm⁻¹ in a 14 cm horizontal gel apparatus with 0.5 μg ethidium bromide per ml.

**Southern blots.** The above gels were blotted onto positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany) via upward capillary transfer and baked for 3 h at 80° C. The *BamHl* fragment from purified pDM2533 containing *E. stewartii hrpL* was labeled with the DIG (Boehringer Mannheim, Mannheim, Germany) kit as described above and hybridization was carried out at 68° C overnight according to the manufacturer’s instructions as described for northern blots. Color development was performed by incubating the membrane in the Promega (St. Louis, MO) substrate for
alkaline phosphatase as described above. For characterization of the transposon mutagenesis of mutants 3C8 and 3E12, purified genomic DNA (5 µg) from each mutant was digested with HindIII and electrophoresed at 10 V cm⁻¹. These gels were blotted as described above and the 2.2 kb HindIII fragment of plasmid pUTmini-Tn5Km, containing the transposon ends and kanamycin resistance gene, was labeled with DIG as described above. Hybridization and color development were carried out as described above.

**Arabidopsis GUS histochemical staining.** Seeds of transgenic Arabidopsis (ecotype Columbia), containing a chimeric reporter gene made up of an *A. thaliana* PR gene (BGL2) promoter and the coding region of β-glucuronidase (GUS) were germinated on Whatman #3 filter paper impregnated with either distilled water, 0.25 strength modified Hoagland’s solution, INA (50 µg ml⁻¹) or E278Ar (suspension of 2 x 10⁹ CFU ml⁻¹ in 0.25 strength Hoagland’s solution. Expression of GUS activity in transgenic Arabidopsis plants was determined histochemically by staining whole leaves of seven day old seedlings germinated on each treatment. Twelve randomly selected seedlings per treatment were stained overnight at 37°C. The staining solution contained 0.5 mg ml⁻¹ of 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) in 0.1 M Na₂HPO₄ (pH 7.0), 10 mM Na₂EDTA, 0.5 mM potassium ferricyanide/ferrocyanide, and 0.06% Triton X-100 (Jefferson et al., 1987). After staining, leaves were cleared in 75% ethanol overnight with two changes of ethanol, then observed for the development of blue color in the leaves.

**Hypersensitive response assays.** To determine whether E278Ar or mutants could induce the hypersensitive response in tobacco (*Nicotiana tabacum* L. cv. ‘Wisconsin’), cultures were grown either 18 h or 48 h in inducing medium as described in
Chapter 2. Cultures were adjusted to an approximate concentration of $5 \times 10^8$ CFU ml$^{-1}$ and infiltrated into panels of tobacco leaves. To determine whether CFEs from these cultures could also induce the HR, CFEs were prepared from 18 h and 48 h cultures via the sonication and boiling method described above. These CFEs were adjusted to a total protein concentration of 0.5 mg ml$^{-1}$ and were infiltrated into tobacco panels. Cultures of DC 283(pRF205) (Wild type E. stewartii with hrpS driven by the pLAFR3 lac promoter) and DM3020 (hrp cluster deletion) (Coplin et al., 1992) were used as positive and negative controls, respectively.

Results

Efficacy of cell free extracts from E278Ar for inducing systemic resistance. Root treatment with a cell-free extract (CFE) from E278Ar, prepared by sonication and boiling, reduced the mean severity of bacterial leaf spot in four experiments from 3.5 to 2.6 (Table 3.2). This was significant at $P = 0.05$ and was equivalent to that observed on plants treated with live cells of E278Ar (mean disease severity rating of 2.5). Treatment of the CFE with protease for 1 h consistently increased disease severity to 3.0. This was an intermediate value between the disease severity observed with controls and non-protease treated CFE. In separate experiments (Table 3.3), plants treated with CFE that had been dialyzed for 48 h had a mean disease severity of 2.7, which was not significantly different ($P = 0.05$) from the mean disease severity on undialyzed CFE (2.8) or live cells of E278Ar (2.5). Mean disease severity for all of these treatments was significantly ($P < 0.05$) lower than that on control plants (3.4). Plants treated on the roots with an extract prepared from E. coli had a mean disease severity of 3.1. (Table 3.3).
<table>
<thead>
<tr>
<th>Root Treatment(^a)</th>
<th>(Xca)</th>
<th>Mean Disease Severity(^b)</th>
<th>Populations of (Xca) (^c) (log CFU/g fresh wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>1.0</td>
<td>&lt;1.5</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>3.5</td>
<td>8.8</td>
</tr>
<tr>
<td>E278Ar</td>
<td>+</td>
<td>2.6</td>
<td>7.2</td>
</tr>
<tr>
<td>CFE from E278Ar</td>
<td>+</td>
<td>2.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Protease-treated CFE</td>
<td>+</td>
<td>3.0</td>
<td>7.6</td>
</tr>
<tr>
<td>LSD(_{0.05})(^d)</td>
<td>0.5</td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\)Roots were treated three days after germination with 200 µl of autoclaved 0.25 strength modified Hoagland's solution (control), with 200 µl of a 1 x 10⁹ CFU ml⁻¹ suspension of E278Ar in 0.25 strength modified Hoagland's solution (E278Ar) or with 200 µl of a cell-free extract from E278Ar prepared by sonication for 10 min followed by boiling for 10 min, centrifugation and filtration, or with a CFE that had been treated with Sigma Protease IV (100 µg) for 1 hr at 37° C (Protease-treated CFE). Plants were challenge-inoculated with a 10⁸ CFU/ml suspension of \(Xca\) seven days following treatment with the biocontrol agent or the CFE.

\(^{b}\)Values represent the mean disease severity ratings for four experiments (two in the case of roots treated with protease-treated CFE). True leaves (five replicates of five plants each per experiment) were rated for disease symptoms 6-8 days following challenge-inoculation with \(Xca\) using a disease scale in which 1 = symptomless; 2 = fewer than three lesions/leaf; 3 = more than three lesions/leaf, less than 25% leaf area affected by disease; 4 = lesions covering 25-50% of the leaf surface area; 5 = lesions covering more than 50% of the leaf surface area and 6 = one or more leaves dead.

\(^{c}\)Populations of \(Xca\) were determined by grinding the foliage of five plants per treatment in 0.25 modified Hoagland's and dilution plating on sucrose-peptone agar amended with streptomycin (50 µg/ml). Data shown are means of two experiments. The detection limit for \(Xca\) was 30 CFU/g fresh weight leaf tissue.

\(^{d}\)Least Significant Difference (\(P=0.05\)) according to Fisher's method.

Table 3.2. Suppression of bacterial spot of radish seedlings by root treatment with a cell free extract (CFE) from \(P.\ agglomerans\) E278Ar grown in L-broth and partial loss of suppression in CFEs treated with protease.
Root Treatment\textsuperscript{a} & \textit{Xca} & Mean Disease Severity\textsuperscript{b} \\
\hline
Control & - & 1.0 \\
Control & + & 3.4 \\
E278Ar & + & 2.5 \\
CFE from E278Ar & + & 2.8 \\
Dialyzed CFE from E278Ar & + & 2.7 \\
CFE from HB101 & + & 3.1 \\
LSD\textsubscript{0.05}\textsuperscript{c} & & 0.6 \\
\hline
\textsuperscript{a}Roots were treated three days after germination with 200 µl autoclaved 0.25 strength modified Hoagland's solution (Control), with 200 µl of a 10^9 CFU/ml suspension of E278Ar in 0.25 modified Hoagland's solution, with a CFE from E278Ar or from \textit{E. coli} HB101, or with a dialyzed (48 hr against water, 10,000 MW cutoff membrane) CFE from E278Ar. Plants were challenge inoculated with a 10^8 CFU/ml suspension of \textit{Xca} seven days following treatment with the biocontrol agent.

\textsuperscript{b}Values represent the mean disease severity ratings for two experiments. True leaves (five replicates of five plants each per experiment) were rated for disease symptoms 6-8 days following challenge inoculation with \textit{Xca} using a disease scale in which 1 = symptomless; 2 = fewer than three lesions/leaf; 3 = more than three lesions/leaf, less than 25% leaf area affected by disease; 4 = lesions covering 25-50% of the leaf surface area; 5 = lesions covering more than 50% of the leaf surface area and 6 = one or more leaves dead.

\textsuperscript{c}Least Significant Difference (\textit{P}=0.05) according to Fisher's method.

Table 3.3. Efficacy of a cell free extract from E278Ar in suppressing of bacterial leaf spot of radish in comparison with a CFE from \textit{E. coli} and dialyzed CFE from E278Ar.
On radish seedlings treated with CFE at concentrations of 0.5 and 0.25 mg ml\(^{-1}\) total protein, the mean disease severity was 2.6 (Table 3.4). This was significantly \((P < 0.05)\) less severe than the controls (mean disease severity of 3.4). Plants treated with CFE diluted to 0.1 and 0.05 mg ml\(^{-1}\) total protein had a mean disease severity of 3.2, which was not significantly different from the controls (Table 3.4). These results were consistent among two experiments.

**Efficacy of cell free extracts from mutants.** The mean disease severity on radish seedlings treated with the live mini-Tn\(^5\)Km mutants (3.2 for 3C8, 3.3 for 4C12) was significantly \((P = 0.05)\) higher than that on the plants treated with E278Ar (2.5). Control plants had a mean disease severity of 3.6, which was not significantly different \((P = 0.05)\) from that on 4C12-treated plants, but was significantly different from 3C8 and wild-type E278Ar (Table 3.5). When cell-free extracts were applied to roots, the mean disease severity on the radish seedlings was 2.6 for CFEs from both mutants. This was again significantly below the disease severity observed on controls and not significantly different from that on plants treated with E278Ar cells. These results were consistent among two experiments.

**Proteins in CFEs.** The SDS-PAGE protein profiles of CFEs prepared from wild type E278Ar and the non-inducing mutants were indistinguishable (Figure 3.1). When Western blots were done with CFEs prepared from wild type E278Ar using antibodies to harpin\(_{Es}\), no reactions were observed (Figure 3.2). However, a positive control CFE from an *E.coli* strain producing harpin\(_{Es}\) produced a strong reaction.
<table>
<thead>
<tr>
<th>Initial E278Ar Dose&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Xca</th>
<th>Mean Disease Severity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Control +</td>
<td>+</td>
<td>3.4</td>
</tr>
<tr>
<td>CFE, 0.5 mg ml&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>+</td>
<td>2.6</td>
</tr>
<tr>
<td>CFE, 0.25 mg ml&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>+</td>
<td>2.6</td>
</tr>
<tr>
<td>CFE, 0.1 mg ml&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>+</td>
<td>3.2</td>
</tr>
<tr>
<td>CFE, 0.05 mg ml&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>+</td>
<td>3.2</td>
</tr>
<tr>
<td>LSD&lt;sub&gt;0.05&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Roots were treated three days after germination with 200 µl of autoclaved 0.25 strength modified Hoagland's solution (control) or with CFE prepared from E278Ar and adjusted to the indicated total protein concentration. Plants were challenge-inoculated on the foliage with a 10<sup>8</sup> CFU ml<sup>-1</sup> suspension of Xca seven days following treatment with the biocontrol agent.

<sup>b</sup>True leaves (five replicates of five plants each per experiment) were rated for disease symptoms 6-8 days following challenge inoculation with Xca using a disease scale in which 1 = symptomless; 2 = fewer than three lesions/leaf; 3 = more than three lesions/leaf, less than 25% leaf area affected by disease; 4 = lesions covering 25-50% of the leaf surface area; 5 = lesions covering more than 50% of the leaf surface area and 6 = one or more leaves dead.

<sup>c</sup>Least Significant Difference (P=0.05) according to Fisher's method.

Table 3.4. Suppression of bacterial leaf spot in radishes by root treatment with varying concentrations of a CFE from E278Ar.
<table>
<thead>
<tr>
<th>Root Treatment(^a)</th>
<th>(Xca)</th>
<th>Mean Disease Severity(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>3.6</td>
</tr>
<tr>
<td>E278Ar cells</td>
<td>+</td>
<td>2.5</td>
</tr>
<tr>
<td>3C8 cells</td>
<td>+</td>
<td>3.2</td>
</tr>
<tr>
<td>4C12 cells</td>
<td>+</td>
<td>3.3</td>
</tr>
<tr>
<td>CFE from 3C8</td>
<td>+</td>
<td>2.6</td>
</tr>
<tr>
<td>CFE from 4C12</td>
<td>+</td>
<td>2.6</td>
</tr>
<tr>
<td>LSD(_{0.05})^c</td>
<td></td>
<td>0.4</td>
</tr>
</tbody>
</table>

\(^a\)Roots were treated three days after germination with 200 \(\mu\)l of autoclaved 0.25 strength modified Hoagland’s solution (control), with E278Ar or mutant cells added at an initial dose of 2 \(\times\) \(10^9\) CFU per root, or with CFEs prepared from mutants and adjusted to a concentration of 0.5 mg ml\(^{-1}\). Plants were challenge inoculated on the foliage with a \(10^8\) CFU ml\(^{-1}\) suspension of \(Xca\) seven days following treatment with the biocontrol agent.

\(^b\)True leaves (five replicates of five plants each per experiment) were rated for disease symptoms 6-8 days following challenge inoculation with \(Xca\) using a disease scale in which 1 = symptomless; 2 = fewer than three lesions/leaf; 3 = more than three lesions/leaf, less than 25\% leaf area affected by disease; 4 = lesions covering 25-50\% of the leaf surface area; 5 = lesions covering more than 50\% of the leaf surface area and 6 = one or more leaves dead.

\(^c\)Least Significant Difference (\(P=0.05\)) according to Fisher’s method.

Table 3.5. Suppression of bacterial leaf spot of radish on plants by root treatment with CFEs prepared from mutants with reduced ability to induce systemic resistance.
Figure 3.1. PAGE of CFE proteins prepared from wild-type E278Ar (wt) and two mutants unable to fully induce systemic resistance.

CFEs were as effective in inducing resistance as that from the wild type. CFEs were prepared by sonication and boiling, then electrophoresed on a 12% separating polyacrylamide (5% stacking) gel, then stained with Coomassie blue.
Figure 3.2. Dot blot of CFEs from wild-type E278Ar probed with anti-harpin$_{Es}$

(A) *E. coli* strain BL2(DE3/pMA2), containing *E. stewartii* hrpN, (B) E278Ar and (C) HB101 at concentrations of 0.5, 0.25, 0.05, and 0.01 mg ml$^{-1}$ total protein. Primary antibodies: anti-harpin$_{Es}$. Secondary antibody: anti-IgG - alkaline phosphatase conjugate.
Likewise, no reaction with anti-harpin$_{Es}$ was observed with CFEs prepared from the mutants (data not shown). These results were consistent among three experiments.

**Genes activated by treatment with chemicals and E278Ar.** Northern blots showed heavy production of PR-1 by plants treated on the roots with INA (50 μg ml$^{-1}$ per root) (Figure 3.3). No PR-1 was detected in leaves of radish seedlings treated on the roots with E278Ar, or control plants. Slight levels of PR-1 expression were seen in RNA from plants infected with E278Ar in one of three blots. In studies of the activation of the ß-1,3-glucanase BGL2-GUS fusion in *A. thaliana*, all twelve seedlings germinated on filter paper treated with INA were stained completely blue, indicating strong GUS activity. None of the seedlings that were germinated on water, modified Hoagland’s solution, or the suspension of E278Ar showed any GUS activity at all.

**PCR on E278Ar and Southern blots.** When PCR was run under conditions of low stringency (low annealing temperature), a band migrating at the predicted length for *hrpL* (about 320bp) was observed in all of the samples tested (Figure 3.4). However, when the annealing temperature of the reaction was increased, the products from *E. coli* and the *Δhrp* mutant of *E. stewartii* were no longer visible, although faint bands were observed with E278Ar, wild type *E. stewartii* and *E. herbicola pv. gypsophilae* genomic DNA (Figure 3.5). These products were all about the same length (about 320 bp) with the exception of the *apaA3* insertion mutant DM782, which showed a larger band. Uncut pDM2533 (*E. stewartii hrpL*) was also able to serve as template for these primers. When the PCR products were blotted and probed with *hrpL* from *E. stewartii*, a faint hybridization was detected with E278Ar. In one blot, *E. coli* strain DH5α gave a very
weak signal. DC283 also gave a weak signal. The PCR product from DM557, the *E. herbicola* pv. *gypsophilae* (*Ehg*) strain to which the primers were designed, gave the brightest signal except for purified *E. stewartii* *hrpL* positive controls (Figures 3.6 and 3.7). On Southern blots carried out to determine the number of insertions of mini-Tn5 in mutants 3C8 and 3E12, the labeled transposon probe hybridized to one band in each mutant, of approximate sizes 23000 bp (3E12) and 21000 bp (3C8) as determined using Kodak Digital Science 1D imaging software (data not shown).

**Induction of the hypersensitive response.** Living cells of E278Ar were able to induce the hypersensitive response in tobacco, but they were much more effective at this induction when taken from cultures that were 48 h old as opposed to 18 h (Table 3.6). E278Ar induced a strong (100% of panel collapsed) HR when older cultures were used, and a weak HR (no HR at all in one of three experiments) when overnight cultures were used. CFEs of E278Ar were also able to induce a strong HR when they were made from 48 hr cultures, but not when made from overnight cultures (Table 3.6). The mutants which had lost efficacy in inducing resistance were unable to induce a strong HR. CFEs from these mutants were able to induce an HR when they were made from 48 hr cultures.

**Discussion**

As previously noted, elicitors from *Phytophthora* and harpins from bacteria induce systemic resistance in plants (Kamoun et al., 1993; Strobel et al., 1996; Jin et al., 1997). Preliminary studies showed that harpin from *Erwinia stewartii* induces resistance in our radish bioassay (Chapter Two). Extracts from E278Ar were prepared using a procedure
Treatment\(^a\) & Hypersensitive response\(^b\) (percent panel collapsed) \\
\hline
IM (negative control) & 0 \\
DM3020 (negative control) & 5 \\
DC283(pRF205) (positive control) & 100 \\
E278Ar, 18 h & 15 \\
E278Ar, 48 h culture & 100 \\
CFE from E278Ar, 18 h culture & 30 \\
CFE from E278Ar, 48 h culture & 100 \\
3C8, 48 h culture & 25 \\
4C12, 48 h culture & 10 \\
CFE from 3C8, 48 h culture & 100 \\
CFE from 4C12, 48 h culture & 100 \\
\hline
\(^a\)Tobacco leaves were infiltrated with either inducing medium, cell suspensions (grown in inducing medium) adjusted to about $5 \times 10^8$ CFU ml\(^{-1}\) in IM, or CFEs from cultures prepared by sonication and boiling. Tobacco plants were between four and five months old at the time of infiltration and kept in the greenhouse.

\(^b\)Percent of the infiltrated area that exhibited a hypersensitive response. Values represent the mean of five leaves, and the experiment was repeated twice with similar results.

Table 3.6. Differential induction of the hypersensitive response in tobacco by cells of and extracts from E278Ar and mutants.
Figure 3.3. Northern blot of RNA isolated from radish leaves and probed with *A. thaliana* PR-1.

(A) Hybridization control (probe DNA), (B) INA treated plants, (C) plants treated with E278Ar, (D) Control plants. Total RNA was isolated from all plants seven days following treatment of the roots.
Figure 3.4. *hrpL* PCR products produced under low stringency (50°C annealing temperature).

Lane 1: E278Ar genomic DNA. Lane 2: purified *E. stewartii hrpL* cloned in pBluescript (pDM2533). Lane 3: DM3020 (Δ*hrpL*) genomic DNA. Lane 4: DM557 (*Ehg*) genomic DNA. Lane 5: DM782 (hrpL::AphA3) genomic DNA. Lane 6: *E. coli* DH5α genomic DNA.

Figure 3.5. *hrpL* PCR products produced under high stringency (58°C annealing temperature).

Lane 1: E278Ar genomic DNA. Lane 2: DC283 (*E. stewartii*) genomic DNA. Lane 3: DM782 (hrpL::AphA3) genomic DNA. Lane 4: *E. coli* DH5α genomic DNA. Lane 5: DM3020 (Δ*hrpL*) genomic DNA. Lane 6: DM557 (*Ehg*) genomic DNA. Lane 7: pDM2533, uncut (*E. stewartii hrpL*)
Figure 3.6. Southern blot of hrpL amplicons from high stringency PCR using genomic DNA as template and probed with E. stewartii hrpL.


Figure 3.7. Second Southern blot, as above.

Lane 1: pDM2533. Lane 2: DM557 (Ehg). Lane 3: DM3020. Lane 4: DM782. Lane 5: E. coli DH5 α. Lane 6: DC283. Lane 7: E278Ar.
based on that used for the isolation of harpin from *E. stewartii* (Ahmad, 1996) and *E. amylovora* (Wei et al., 1992). The fact that the activity was retained within the dialysis bag with a 10,000 molecular weight cutoff (Table 3.3) indicates that it was due at least in part to a large molecule or a set of large molecules. This elicitor or elicitors were not present in CFE prepared from *E. coli* (Table 3.3). The reduction of efficacy of cell-free extracts by protease treatment of (Table 3.2) suggests that at least part of the activity in the extracts was due to a protein. The activity also withstood boiling for 10 min, suggesting that a heat-stable protein is involved. Both living cells of E278Ar and the CFE were able to induce an HR in tobacco. The observation that the HR was induced more strongly by older cultures and CFEs indicates that the ability to induce an HR is under some kind of growth phase regulation. Cultures of that age are in stationary phase and are physiologically different from log-phase cultures. The fact that CFEs from younger cultures were not strong inducers of HR indicates that HR induction by older cultures depends on the stationary phase metabolism of the bacteria, not merely old cells leaking their contents. However, CFEs from 18 h cultures were able to induce resistance in the radish bioassay (Chapter 2). This is further evidence for the presence of multiple elicitors of resistance in the CFE, presumably including some which do not also induce HR. *hrp* gene clusters are also under the control of signals from the plant and the environment and generally require minimal media mimicking the apoplast for full activation (Rahme et al., 1992; Wei et al., 1992; Huynh et al., 1989). This, however, would seem to argue against *hrp* gene expression in the rhizosphere, which is considered to be a fairly nutrient-rich environment (Curl and Truelove, 1982).

Whether the protein elicitor produced by E278Ar is secreted remains unknown. However, the behavior of the mutants may offer some clues. The two mutants were
unable to induce resistance as well as the wild type (Table 3.5), even though their extracts were just as active as live E278Ar cells (Table 3.5) or CFE prepared from E278Ar. This suggests that the mutants were able to produce the elicitor(s), but could not deliver it to the plant. These mutants colonized the roots as quickly as the wild type and reached populations comparable to E278Ar on roots. Reduced ability to colonize and grow on radish roots, therefore, is not a likely explanation for the reduced efficacy of the mutants. A deficiency in secretion seems a more likely explanation, especially as secretion pathways offer a large target for transposon mutagenesis (Alfano and Collmer, 1996; Beer et al., 1991). The fact that the total protein profiles of the mutant extracts do not differ from the wild type extract (Figure 3.1) also is consistent with the hypothesis that the mutants still produce the elicitor.

Attempts to locate elicitor activity in culture filtrates were unsuccessful (data not shown). Furthermore, protein gels of culture filtrates, even culture filtrates concentrated several hundred times, showed no extracellular proteins. This suggests that if a protein was secreted by E278Ar, it may have been either kept within the envelope or translocated directly into plant cells. These are hallmarks of the Type III secretion system encoded by the *hrp* clusters of pathogenic bacteria, which are large aggregates of proteins that create a channel to inject proteins directly across both bacterial membranes and a plant or animal membrane/wall (Alfano and Collmer, 1996; Bogdanove et al., 1996; Bonas, 1994; Huang et al., 1992). To date, type III secretion systems are considered to be present in pathogens only, but cloning *hrp* gene clusters can enable nonpathogens to elicit hypersensitive responses. For example, cloning the *Pseudomonas syringae* pv. *syringae* *hrp* cluster into *Pseudomonas fluorescens* enables *P. fluorescens* to elicit an HR in tobacco (Huang et al., 1988). There is also a precedent for mobile *hrp* genes in nature. The *hrp*
cluster of *Erwinia herbicola* pv. *gypsophilae* resides on a plasmid which enables pathogenic strains to cause tumors (Nizan et al., 1997). It may not be unreasonable to expect to find partial or nonfunctional *hrp* clusters in organisms like E278Ar which induce the hypersensitive response in tobacco (Table 3.6) and systemic resistance, and yet do not appear to be pathogens.

The elicitor from E278Ar was heat stable and induced the HR on tobacco. These are also properties of the harpin family of proteins which are the HR inducers of plant pathogenic bacteria (Wei et al., 1992). This suggests the elicitor from E278Ar might be similar to harpin. When Western blots of CFEs from E278Ar were done using anti-harpin<sub>E<sub>E</sub></sub> antibodies, no cross-reactions were observed (Figure 3.2). However, proteins other than harpin may be secreted through the *hrp* system. Several *avr* gene products are believed to be delivered to plant cells through the *hrp* pathway, including *avrRpt2* and *avrRpm1* from *P. syringae* pv. *maculicola* (Ritter and Dangl, 1996; Reuber and Ausubel, 1996), the AvrBs3 family in *Xanthomonas* (Yang and Gabriel, 1995) and, presumably, various Avr proteins interacting with cytoplasmic *R* gene products (Bent et al., 1994; Bent, 1996; Mindrinos, et al., 1994; Grant et al., 1994). It is possible that the protein present in the CFE from E278Ar was not a harpin at all, even if it were secreted through a type III secretion pathway. Again, the inability to find the protein elicitor in the culture filtrate is consistent with the known behavior of *avr* gene products (Brown et al., 1993; Young et al., 1994).

Although the living cells of E278Ar and extracts from E278Ar and non-inducing mutants were able to elicit both the hypersensitive response and induced systemic resistance, they did not induce a buildup of PR-1 in radish (Figure 3.3) or increased
BGL2-GUS activity in Arabidopsis. This suggests that the SA-dependent pathway did not lead to resistance induced by E278Ar. Perhaps the ISR pathway was induced (Pieterse, 1998). Alternatively, E278Ar could condition plants to respond more quickly when infection occurs, as is the case with plants grown in some suppressive compost potting mixes (Zhang et al., 1998).

When dilutions of the CFE from E278Ar were applied to radish roots, a minimum concentration was reached below which it was not active (Table 3.4). This agrees with the findings in Chapter 2 that show there is also a threshold concentration of live cells that was needed for effective induction of systemic resistance. It is interesting to note that when the CFEs were diluted, they completely lost their ability to suppress bacterial leaf spot, whereas when they were treated with protease, they retained some activity (Table 3.2). This suggests that a threshold concentration is required for activity of the putative non-protein (or protease-resistant) elicitor(s) that are also present in the CFE.

The PCR reaction using primers designed to Ehg hrpL sequences produced ambiguous results. hrpL was chosen because it is a sigma factor regulating the expression of the hrp regulon (Wei and Beer, 1995). Therefore, it would likely be conserved if any part of a hrp cluster were present in E278Ar. However, sigma factors share conserved regions (Morrett and Segovia, 1993) so it is also possible that primers to the Ehg hrpL would amplify sequences common to other sigma factors. This indeed seems to be the case when the reaction is run under low stringency (Figure 3.4). However, under high stringency, only the Erwinia and Pantoea templates yielded PCR products (Figure 3.5). Does this mean that hrpL actually was produced by E278Ar? The evidence is still very equivocal. Cloning and sequencing of the PCR products will probably be necessary in
order to determine this for certain. The Southern blots (Figures 3.6 and 3.7) of the PCR gels are also equivocal, as in one case a faint band appears for *E. coli*. In this case, it may even be possible that *hrpL* from *E. stewartii* can hybridize very weakly to another sigma factor in *E. coli*.

The extract contained an elicitor that was heat-stable, of high molecular weight and sensitive to protease. However, protease treatment did not completely destroy the activity of the extract, which may suggest that there was more than one elicitor present in the extract. Mutants that were unable to fully induce resistance when applied to roots were still able to serve as sources of fully effective extracts. Since there is a protein in the extracts that was inducing systemic resistance and also an HR, PCR was undertaken to see whether E278Ar might have at least parts of a *hrp* system. Initial results seem to indicate that it might indeed have *hrpL*, but this has yet to be conclusively demonstrated.

In summary, this work showed that a cell-free extract, prepared from E278Ar, was as effective in inducing systemic resistance in radish to bacterial leaf spot as were living cells or chemical inducers. Although the cells and extracts induced both the hypersensitive response and systemic resistance, they did not induce an accumulation of PR-1 in radishes or an increase in BGL2-GUS activity in Arabidopsis. This suggests that E278Ar induces a mechanism other than SA-mediated SAR.
CHAPTER 4
SUMMARY

This research on systemic resistance induced in plants by compost-amended potting mix demonstrated that microorganisms in these mixes were required for this effect. The mix contained many bacteria and fungi that can suppress soilborne plant pathogens (Hoitink et al., 1997). Although several of these microorganisms can be excellent biocontrol agents, most were not able to induce systemic resistance in cucumber. This may partially explain the observation that many compost-amended mixes that suppress soilborne diseases do not suppress foliar diseases.

It was determined that *P. agglomerans* strain E278A and *T. hamatum* 382 induced a significant degree of systemic resistance in cucumber against anthracnose and in radish against bacterial leaf spot. It had been established that these biocontrol agents also effectively control damping-off caused by *Rhizoctonia solani* and *Pythium ultimum* (Kwok et al., 1987; Chen et al., 1987; Hoitink, unpublished data). The systemic resistance induced by E278A appeared to differ from that mediated by SA because PR proteins were not activated in radish or *A. thaliana* by root treatment with this biocontrol agent. Both E278A and its spontaneous rifampicin-resistant mutant, E278Ar were effective inducers of systemic resistance and effective root colonizers on radish. The foregoing suggests that it may be possible to develop inoculants which consistently both induce systemic resistance
in plants and provide consistent control of diseases caused by a broad spectrum of soilborne plant pathogens.

A novel growth pouch bioassay was developed for assessing the ability of microorganisms to induce systemic resistance in radish against bacterial leaf spot. This new bioassay had a number of advantages over earlier bioassays using cucumber or Arabidopsis. These advantages included miniaturization, which allows for greater replication, greater ease of inoculation, and a shorter test period (14-18 as opposed to 25-30 days).

Spatial separation between the biocontrol agent on the roots and the pathogen on the leaves is required to allow one to draw conclusions about the systemic nature of the resistance induced. This was accomplished in this new bioassay. Like in earlier bioassays (Pieterse et al., 1997; Leeman et al., 1995a) differences in the chemical and physical properties of the soil were minimized so that the impacts of specific biocontrol agents could be studied more closely. Because these growth pouches could be sterilized prior to use and seeds could be surface-sterilized, competition from other microorganisms was minimized. In spite of all these advantages, the growth pouch bioassay did suffer from the same variability problems that plague all bioassay systems. Finally, the new bioassay also was useful for evaluating the efficacy of putative chemical inducers of systemic resistance, such as harpin or INA.

The growth pouch assay was used successfully to screen mini-Tn5Km-induced mutants of E278Ar for a loss of their ability to induce systemic resistance. Three mutants were identified that had reduced abilities to induce resistance.
Cell-free protein extracts (CFEs) that were made from E278Ar and two non-inducing mutants, prepared by the same method used to prepare harpin from pathogens (Wei et al., 1992), were as effective in inducing systemic resistance as living cells of wild-type E278Ar, INA, or harpinEs. This suggests that E278Ar made an elicitor of induced systemic resistance. CFEs lost part of their ability to induce resistance after treatment with protease, indicating that a protein may have been responsible for at least part of the activity. The activity was heat-stable, indicating that the elicitor may be a harpin-like protein because harpins are also heat-stable and can induce systemic resistance (Wei et al., 1992). The CFE from E278Ar did induce the hypersensitive response in tobacco, but it did not contain a protein that cross-reacted with anti-harpinEs antibodies.

Because living cells of E278Ar could induce HR in tobacco, this organism might be a minor pathogen or have some of the genes required for pathogenicity. This could also explain its ability to induce systemic resistance in plants (van Loon et al., 1998) even though it did not cause symptoms of disease on the roots of radish seedlings. hrpL is a regulatory gene that is found in all Pseudomonas and Erwinia/Pantoea hrp clusters. Assuming that this might be indicative of the presence of hrp genes, we tested for the presence of hrpL in E278Ar. PCR primers were designed to the coding region of hrpL from the closely related bacterium Erwinia herbicola pv. gypsophila. (Ehg). A weak band corresponding to hrpL in Ehg indeed was found in E278Ar, and it hybridized weakly to a labeled hrpL probe. This suggests that some hrp genes may have been present in E278Ar and that this, therefore, might offer an explanation for some of its ability to induce systemic resistance.
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