AN INTEGRATED MANAGEMENT STRATEGY FOR BACTERIAL SPECK DISEASE OF TOMATO IN NEW YORK: DECIPHERING THE WEB OF INDUCED RESISTANCE

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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May 2008

AN INTEGRATED MANAGEMENT STRATEGY FOR BACTERIAL SPECK DISEASE OF TOMATO IN NEW YORK: DECIPHERING THE WEB OF INDUCED RESISTANCE

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Cornell University 2008

Incorporating plant activators into integrative pest management programs is an appealing option for controlling bacterial diseases of greenhouse and field-grown tomatoes. Two types of plant activators, compounds that control disease without directly impacting pathogens, were evaluated for effects on tomato defense gene activation, disease control and yield. The two plant activators involve different signaling pathways; one induces systemic acquired resistance (SAR) and a second activates induced systemic resistance (ISR).

In greenhouse assays, acibenzolar-*S*-methyl (ASM, SAR-inducing compound) effectively reduced bacterial speck incidence and severity, both alone and with the ISR-inducing compound (mixture of two *Bacillus* spp. known as plant growth-promoting rhizobacteria, PGPR). Elevated activation of salicylic acid (SA) and ethylene (ET) pathways was observed following ASM application. The PGPR compound provided inadequate disease control and inconsistently modified defense gene expression. Combining the two activators did not involve negative cross-talk between signaling pathways as disease control was on par with or better than ASM alone. Based on these

results, ASM appears to be a viable option for bacterial speck management in greenhouse tomato transplant production.

Defense gene activation in three field-grown tomato cultivars via ASM was evaluated without pathogen pressure. Quantitative real-time PCR analysis following two ASM applications revealed that ASM induced the SA and ET, but not jasmonic acid (JA), pathways in all cultivars tested. Gene expression in all three cultivars responded with higher activation following the second ASM application (applied seven days after the first).

ASM, PGPR and copper were evaluated for control of bacterial speck and tomato defense gene activation over three field seasons. ASM controlled *Pseudomonas syringae* pv. *tomato* as well as copper with no negative effect on yield. The PGPR compound reduced bacterial speck symptoms though provided inconsistent control and no priming of signaling pathways was observed. Alone or in combination with ASM, the PGPR compound provided some yield boost in one of three years. All treatments negatively impacted pathogen growth. Response of ASM-treated plants was dependant on disease pressure; SA and ET pathways were activated to detectable levels only under high disease pressure. Implications of these findings on management strategies and defense pathway interactions are discussed.

BIOGRAPHICAL SKETCH

Maryann A.B. Herman was born Maryann A. Borsick in Clarence, New York on September 27, 1979 to Adel and Joseph Borsick. Upon graduation from Clarence Senior High School in 1997, she studied Biology at Allegheny College in Meadville, Pennsylvania. During her four years at Allegheny, Maryann studied tropical ecology in Ecuador, investigated differentiation signals in *Dictyostelium discoideum*, and completed an undergraduate thesis examining the effect of selection for disease resistance on host enzyme activity. After earning her Bachelor of Science degree in 2001, she spent two years as a Research Technician II in the Medical School and Biochemistry Department of the State University of New York at Buffalo investigating regulation of Na, K-ATPase via prostaglandins. In the summer of 2003, Maryann began her Ph.D. at Cornell University in the Department of Plant Pathology. She joined the laboratory of Dr. Christine D. Smart at the New York State Agricultural Experiment Station in Geneva, NY. In Dr. Smart's program, Maryann used applied and basic approaches to examine induced defense responses in tomatoes. More specifically, her thesis research focused on compounds that induce plant signaling pathways and how they influence plant-pathogen interactions in the field. Findings of this research provide recommendations for improved management strategies for bacterial disease control in tomato production.

I would like to dedicate my doctoral thesis to my husband, Mike. His love and support have kept me grounded throughout this tremendous undertaking. His ability to make me laugh despite seemingly catastrophic experimental failures and enduring faith in my abilities has fueled my confidence and drive.

I would also like to dedicate this to my parents, Joe and Adel, and my godparents, Sharon and Unk. They have shaped the person I've become and taught me that you can achieve anything through hard work and belief in yourself.

Dr. Silvia Restrepo is a great friend and inspiration as a scientist. She helped mould the early stages of my dissertation and I will be forever grateful for the opportunity to work with her.

ACKNOWLEDGEMENTS

I would like to thank my major advisor, Dr. Christine D. Smart, for her support and guidance throughout all aspects of my graduate career. She has provided many opportunities for me to grow professionally and obtain my career goals. I have been fortunate to have such an inspiring mentor who excels in many different areas of biology and education.

I would also like to acknowledge my committee members Drs. Thomas J. Burr, Thomas Björkman, and Stephen V. Beer. Their collective advice and support has proven invaluable to my research and education.

Finally I would like to acknowledge Amy Andersen for invaluable assistance, Kevin Myers for technical support, John Barnard for statistical support and past and present members of the Smart lab, Holly Lange, Silvia Restrepo, Tanya Taylor, Jennifer Davidson, Sarah Davidson, Ning Zhang, Gabrielle Meeks, and Amara Camp, without whom this work would not have been possible.

This work was funded in part by the New York State IPM and USDA NRI grants programs, Robert M. Gilmer Graduate Student Award and American Phytopathological Society and Cornell Travel Grants.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 PLANT ACTIVATORS

Activating plant defenses against pathogen and insect pests is a wonderfully enticing concept. While induced resistance has been studied for decades in the laboratory, this information has not translated to consistent pest control in the field. Plant activators are compounds that can activate plant signaling and control disease without directly impacting the pathogen. Additionally, many of these products claim to increase plant health and yield and have a lower environmental impact. Plant activators could be a valuable tool in an integrated pest management program by delaying initial pesticide applications or they could be alternated with chemical control. Furthermore, some activators are certified for organic use and could be implemented in organic growing systems. Though several plant activating compounds are commercially available, their utilization by growers is quite limited. This is mainly due to inconsistent effectiveness of such products under field conditions.

Currently, it is not fully known how plant activators interact with target plants and other organisms and how to best implement these products in to commercial production (McSpadden Gardener and Fravel 2002). Fundamental understanding of the molecular mechanisms of induced defense responses and the role of plant activators is critical to implement innovative control strategies in the field.

When under pathogen attack, all plants possess the ability to activate defense responses. Disease occurs when a virulent pathogen is able to evade detection or the effects of active defenses or suppresses plant response (Desender et al. 2007; Kuc 1982; van Loon et al. 1998). Induced resistance involves activation of innate plant defenses via specific stimuli, which in turn enables plants the capacity to respond against later pathogen attack (Vallad and Goodman 2004; van Loon, Bakker, and Pieterse 1998). There are two well characterized induced resistance pathways; each require distinct regulatory pathways, though elicit similar phenotypic responses. Systemic acquired resistance (SAR), which involves salicylic acid signaling, provides one type of induced resistance. Living microbes that activate a resistance mechanism independently of salicylic acid, known as induced systemic resistance (ISR), make up the second type of induced resistance pathway.

1.1.1 Systemic acquired resistance

Induced resistance has been documented in plant-pathogen interactions for over a century (Chester 1933, 1933). It wasn't until the 1960's that SAR, an inducible phenomenon triggered by biotic and abiotic stress or functional analogs of salicylic acid, was clearly demonstrated in the laboratory and field (Cruikshank and Mandryk 1960; Durrant and Dong 2004; Ross 1961, 1961). Activation of SAR confers systemic resistance that is effective against a wide range of pathogens, including bacteria, viruses, fungi and oomycetes (Sticher et al. 1997). Many pathogenesis-related genes (*PR* genes) are activated upon SAR induction, which leads to increased accumulation of

salicylic acid and pathogenesis-related proteins locally and systemically (Durrant and Dong 2004; Nawrath et al. 2006; Schenk et al. 2003).

Acibenzolar-*S*-methyl (ASM) is a commercially available activator derived from benzo[1,2,3]thiadiazole (BTH) and a functional analog of salicylic acid known to induce the SAR pathway (Fidantsef et al. 1999; Kuc 2001; Thaler et al. 1999). Acibenzolar-*S*-methyl effectively activates signaling in a wide range of plants, potentially providing broad-spectrum protection (Oostendorp et al. 2001). Specifically, ASM has been found effective against a wide range of bacterial pests of tomato (Anith et al. 2004; Baysal et al. 2003; Graves and Alexander 2002; Louws et al. 2001; Pradhanang et al. 2005; Wilson et al. 2002). Tomato is one of the few crops where a plant activator is currently recommended to growers for controlling bacterial diseases. This recommendation applies to NY State as well as other states in the Northeast, but the compound is not widely used.

1.1.2 Induced systemic resistance

The phenomenon of ISR is distinguished from SAR as it is dependent on plant responsiveness to jasmonic acid and ethylene signaling but is independent of salicylic acid (Bostock 2005; Kloepper et al. 2004; Persello-Cartieaux et al. 2003; Pieterse et al. 2002; Ryu et al. 2003; Siddiqui 2005). Discovery of ISR was much more recent than SAR, though research over the past twenty-five years has greatly increased our knowledge of this induced signaling pathway (Vallad and Goodman 2004; van Loon, Bakker, and Pieterse 1998). ISR-inducing products are frequently plant growth-promoting rhizobacteria (PGPR), or yield-enhancement biologicals, which claim to boost

yield while reducing disease (Kennedy et al. 2004; Siddiqui 2005). PGPR are living bacteria (many are *Bacillus* or *Pseudomonas* sp.) which can be mixed in with soil just prior to planting seed, or used as a seed treatment (Nakkeeran et al. 2005). The PGPR compound used in these studies contained a mixture of endospores from two species of *Bacillus*, *B. amyloliquefaciens* strain IN937a (an ISR-stimulating strain) and *B. subtilis* strain GB03 (a growth-promoting strain) (Domenech et al. 2006).

Typically, PGPR colonize plant roots and feed off exudates and in turn provide direct and indirect effects on plant growth and disease resistance (Antoun and Prevost 2006; Kennedy, Choudhury, and Kecskes 2004; Nelson 2004; Persello-Cartieaux, Nussaume, and Robaglia 2003; Pieterse et al. 2002). Increasing nitrogen uptake, synthesis of phytohormones, solubilization of minerals, and iron chelation are some of the methods by which PGPR benefit plant growth (Bowen and Rovira 1999). Plant growth-promoting rhizobacteria may also directly suppress some soil-borne pathogens (independently from ISR) via production of siderophores, antimicrobial metabolites, or competing for nutrients and/or niches (Nelson 2004).

PGPR stimulate ISR by activating the formation of physical and chemical barriers in the host which can generate an increase in resistance to foliar pathogens and pests (Persello-Cartieaux et al., 2003; Ryu et al., 2003; Pieterse et al., 2002; Kloepper et al., 2004; Bostock, 2005). This phenomenon has been documented in many plant-insect and plant-pathogen interactions (Zehnder et al., 1997; Zehnder et al., 2001; Conrath et al., 2006; Stout et al., 2006; Tuzun and Bent, 2006). There are several examples of plants treated with PGPR which show a decrease in insect herbivory and/or crop loss

(Boughton et al. 2006; Herman et al. 2007; Kempster et al. 2002; Stout et al. 2002; Zehnder et al. 1997). Additionally, studies have found PGPR to effectively reduce tomato diseases alone, or in combination with SAR-inducing compounds (Anith et al. 2004; Domenech et al. 2006; Guo et al. 2004; Jetiyanon et al. 2003; Murphy et al. 2003).

1.2 DEFENSE GENE ACTIVATION

Plant hormone signaling pathways are frequently associated with defense responses and connected in complex regulatory networks, with salicylic acid, ethylene and jasmonic acid being the key players in induced resistance (Bostock 2005).

Activation and duration of SAR correlates with induction of salicylic acid defense response-related genes, though the role of individual genes in disease resistance remains unclear (Durrant and Dong 2004; Uknes et al. 1992; Ward et al. 1991). Expression of SAR-related genes provides markers to follow SAR activation. Acidic *PR-1* is a marker for the salicylic acid signaling pathway and ASM is known to activate this pathway to induce SAR in tomato (Block et al. 2005; Tornero et al. 1997; van Kan et al. 1992).

Ethylene is thought to enhance and stimulate defense responses (van Loon et al. 2006). The ethylene signaling pathway, as measured by expression of the tomato basic *PR-1* gene, could be used as a marker of ISR (Block et al. 2005; Tornero et al. 1997; van Kan et al. 1992). This gene may be involved in PGPR-plant interaction due to the role of ethylene in expression of ISR.

Induction of the wound-inducible jasmonic acid pathway can be measured by the proteinase inhibitor marker gene *Pin2* (Bowles 1998; Fidantsef et al. 1999; Penacortes et al. 1995). This pathway is activated when a plant is wounded, such as by insect herbivory (Casaretto and Corcuera 1995) and is thought to be involved with ISR (Pieterse et al., 2002; Kloepper et al., 2004; Bostock, 2005).

Priming occurs when plants can respond faster and to a greater degree when challenged by both biotic and abiotic stressors (Conrath et al. 2006). This phenomenon has generally been associated with PGPR, but has been found in SAR-induced plants upon further challenge with pathogens (Beckers and Conrath 2007).

Antagonism and synergy between these three pathways has been reported (Bostock 2005; Lorenzo and Solano 2005). Though in the laboratory setting, activation of ISR (pseudomonad) and SAR (avirulent pathogen) simultaneously led to additive disease control and no apparent cross-talk between defense response pathways (van Wees et al. 2000).

1.3 BACTERIAL SPECK DISEASE OF TOMATO

Pseudomonas syringae pv. *tomato* (Okabe 1933) (Young et al. 1986), a gram-negative member of the Gammaproteobacteria, causes bacterial speck disease of tomato (Jones et al. 1991; Madigan et al. 2003; Pedley and Martin 2003). This disease was originally described in the United States and Taiwan in 1933 (Bryan 1933). Disease development is favored by cool temperatures (18-24°C) and high relative humidity (Jones, Stall, and Zitter 1991). Symptomatic plants typically exhibit small, necrotic lesions surrounded by

chlorotic halos on foliage and fruit. Bacterial speck is a persistent disease that can be economically important when climactic conditions are optimal for disease development (Goode and Sasser 1980). While this disease does not often kill the plant, symptoms on fruit can decrease marketability (Figure 1.1).

Control of *P. syringae* pv. *tomato* is mainly achieved through cultural control methods such as sanitation, use of disease-free seed or hot water treatment, control of weed and insect pests, crop rotation, and water management, or by copper-based bactericides (Reiners and Petzoldt 2007; Smart et al. 2005). Prevention of infection is key; overhead irrigation and mechanical wounding of plants provides a golden opportunity for pathogen infection. Effective gene-for-gene resistance to bacterial speck has been found in plants with the resistance gene *Pto*, though this gene has not yet been bred into all tomato cultivars, specifically fresh-market varieties (Pedley and Martin 2003; Wilson et al. 2002). Unfortunately, *Pto*-containing tomatoes are not resistant to all strains of *P. syringae* pv. *tomato* (Buonaurio et al. 1996; Lawton and Macneill 1986), as races of the pathogen that can overcome this R-gene have been reported.

While copper-based bactericides effectively control bacterial speck in New York, copper-resistant strains have arisen in other locations (Alexander et al. 1999; Bender and Cooksey 1986; Cuppels and Elmhirst 1999; Scheck et al. 1996; Silva and Lopes 1995) and excessive use of heavy metals in agriculture raises environmental concerns (He et al. 2005). Alternative control compounds, such as ASM, have been found to control bacterial speck as well as copper, though there have been some reports of yield reduction (Louws et al. 2001; Stout et al. 1998) as well as increased yield (Pradhanang et al. 2005).

Figure 1.1. Symptoms of bacterial speck disease of tomato.

1.4 OBJECTIVES AND SCOPE OF THE STUDY

Despite decades of laboratory research on plant activators, they fail to be widely used in the field. The objective of this study was to determine the timing and duration of plant responses to activators and learn how to best integrate them into tomato production in New York State. While both ISR and SAR induce the plant's natural defense mechanisms, they operate via different pathways and it is unknown which mechanism will have greater efficacy against pathogens of tomato in NY. To this end, three distinct projects examined gene activation by plant activators and the implications for management of bacterial diseases of tomato.

1.4.1 Induction of plant defense response pathways by plant activators and *Pseudomonas syringae* pv. *tomato* on greenhouse-grown tomatoes

The first objective examined the effectiveness of plant activators in a controlled greenhouse environment utilizing the tomato-*P. syringae* pv. *tomato* pathosystem. While the SAR-inducing compound had been shown to control bacterial speck (Louws et al. 2001), it was not clear whether the ISR-inducing compound would perform as well. Despite the high level of bacterial disease control with ASM, phytotoxicity and yield reduction have been reported following application of an SAR-inducing compound in tomato (Louws et al. 2001; Stout, Brovont, and Duffey 1998). Two reports have documented enhanced disease control by combining the two signaling pathways, therefore it is possible that the growth-promoting effect of the PGPR compound (Kokalis-Burelle et al. 2002) could mitigate the yield-reducing effect of ASM (Anith et al. 2004; Nair et al. 2007).

It is unknown how the combination of plant activators would affect defense gene signaling pathways, though crosstalk between each of these pathways has been documented (Bostock 2005). Furthermore, it has not been examined whether treatment with either activator, alone or in combination, would result a priming effect when challenged with the pathogen.

1.4.2 Defense gene expression patterns of three SAR-induced tomato cultivars in the field

The goal of this study was to follow signaling pathways in the field to determine whether they were induced at the same rate and to the same degree in three tomato cultivars in New York. Cultivar differences have been found to affect the level of induced resistance and degree of insect control from insecticides (Fan et al. 2007; Schuster 1977). This information is crucial to determine the optimal application regime which will enable the highest level of disease control. Results of this study provide fundamental information on the defense signaling involved with use of the plant activator ASM under field conditions.

1.4.3 Defense gene activation over three field seasons

The final portion of this study involved a comprehensive, three year analysis of plant activators and copper for control of bacterial speck disease in the field. This objective was critical to synthesize information from the previous studies and evaluate whether plant activators could be effectively incorporated into a disease management strategy in New York. Additionally, it is completely unknown if the two products used together could act synergistically to enhance both yield and disease control. Additionally,

studying defense pathway activation by monitoring marker gene expression following the application of a plant activator and inoculation with a pathogen is necessary to determine if pathogen attacks trigger the re-activation of plant defenses. These pathogen-plant activator interactions will further enhance our ability to integrate plant activators into disease control strategies in New York.

Within this framework, I have been working to address several distinct issues: (1) Can plant activators be effectively implemented in a disease management strategy? (2) How do plant activators and traditional control measures affect plant defense gene expression, both in the presence and absence of pathogens? (3) How do these products affect pathogen populations living on the plant surface?

By combining the results of all three objectives, this study provides molecular and applied data pertinent to grower management practices. The culmination of this work is an integrated pest management strategy for the control of bacterial speck tomato in New York.

REFERENCES

Alexander, S.A., Kim, S.H., and Waldenmaier, C.M. 1999. First report of copper-tolerant *Pseudomonas syringae* pv. *tomato* in Virginia. *Plant Disease* 83 (10):964.

Anith, K. N., Momol, M. T., Kloepper, J. W., Marois, J. J., Olson, S. M., and Jones, J. B. 2004. Efficacy of plant growth-promoting rhizobacteria, acibenzolar-*S*-methyl, and soil amendment for integrated management of bacterial wilt on tomato. *Plant Disease* 88 (6):669-673.

Antoun, H., and Prevost, D. 2006. Ecology of Plant Growth Promoting Rhizobacteria. In *PGPR: Biocontrol and Biofertilization*, edited by Z. A. Siddiqui. Dordrecht: Springer.

Baysal, O., Soylu, E. M., and Soylu, S. 2003. Induction of defense-related enzymes and resistance by the plant activator acibenzolar-*S*-methyl in tomato seedlings against bacterial canker caused by *Clavibacter michiganensis* ssp *michiganensis*. *Plant Pathology* 52 (6):747-753.

Beckers, G. J., and Conrath, U. 2007. Priming for stress resistance: from the lab to the field. *Current Opinion in Plant Biology* 10 (4):425-431.

Bender, C. L., and Cooksey, D. A. 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato* - conjugative transfer and role in copper resistance. *Journal of Bacteriology* 165 (2):534-541.

Block, A., Schmelz, E., O'Donnell P, J., Jones, J. B., and Klee, H. J. 2005. Systemic acquired tolerance to virulent bacterial pathogens in tomato. *Plant Physiology* 138 (3):1481-1490.

Bostock, R. M. 2005. Signal crosstalk and induced resistance: straddling the line between cost and benefit. *Annual Review of Phytopathology* 43:545-580.

Boughton, A. J., Hoover, K., and Felton, G. W. 2006. Impact of chemical elicitor applications on greenhouse tomato plants and population growth of the green peach aphid, *Myzus persicae*. *Entomologia Experimentalis Et Applicata* 120 (3):175-188.

Bowen, G. D., and Rovira, A. D. 1999. The rhizosphere and its management to improve plant growth. *Advances in Agronomy* 66:1-102.

Bowles, D. 1998. Signal transduction in the wound response of tomato plants. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 353 (1374):1495-1510.

Bryan, M. K. 1933. Bacterial speck of tomatoes. *Phytopathology* 23 (11):897-904.

Buonaurio, R., Stravato, V. M., and Capelli, C. 1996. Occurrence of Pseudomonas syringae pv tomato race 1 in Italy on Pto gene-bearing tomato plants. *Journal of Phytopathology-Phytopathologische Zeitschrift* 144 (9-10):437-440.

Casaretto, J. A., and Corcuera, L. J. 1995. Plant proteinase inhibitors: a defensive response against insects. (Review). *Biol Res* 28 (4):239-49.

Chester, K. S. 1933. The problem of acquired physiological immunity in plants. *Quarterly Review of Biology* 8 (2):129-154.

Chester, K. S. 1933. The problem of acquired physiological immunity in plants (continued). *Quarterly Review of Biology* 8 (3):275-324.

Conrath, U., Beckers, G. J. M., Flors, V., Garcia-Agustin, P., Jakab, G., Mauch, F., Newman, M. A., Pieterse, C. M. J., Poinssot, B., Pozo, M. J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D., Zimmerli, L., Mauch-Mani, B., and Grp, Prime-A-Plant. 2006. Priming: getting ready for battle. *Molecular Plant-Microbe Interactions* 19 (10):1062-1071.

Cruikshank, I.A.M., and Mandryk, M. 1960. The effect of stem infestations of tobacco with *Peronospora tabacina* Adam. on foliage reaction to blue mold. *Journal of Australian Institute of Agricultural Science* 26:369-372.

Cuppels, D. A., and Elmhirst, J. 1999. Disease development and changes in the natural *Pseudomonas syringae* pv. *tomato* populations on field tomato plants. *Plant Disease* 83 (8):759-764.

Desender, S., Andrivon, D., and Val, F. 2007. Activation of defence reactions in Solanaceae: where is the specificity? *Cellular Microbiology* 9 (1):21-30.

Domenech, J., Reddy, M. S., Kloepper, J. W., Ramos, B., and Gutierrez-Manero, J. 2006. Combined application of the biological product LS213 with *Bacillus*, *Pseudomonas* or *Chryseobacterium* for growth promotion and biological control of soil-borne diseases in pepper and tomato. *Biocontrol* 51 (2):245-258.

Durrant, W. E., and Dong, X. 2004. Systemic acquired resistance. *Annual Review of Phytopathology* 42:185-209.

Fan, Z. W., Buschmann, H., Muller-Stover, D., and Sauerborn, J. 2007. Main effects and interactions among acibenzolar-*S*-methyl, a biocontrol fungus and sunflower cultivar on control of *Orobanche cumana* Wallr. *Journal of Plant Diseases and Protection* 114 (2):76-81.

Fidantsef, A. L., Stout, M. J., Thaler, J. S., Duffey, S. S., and Bostock, R. M. 1999. Signal interactions in pathogen and insect attack: expression of lipoxygenase, proteinase inhibitor II, and pathogenesis-related protein P4 in the tomato, *Lycopersicon esculentum*. *Physiological and Molecular Plant Pathology* 54 (3-4):97-114.

Goode, M.J., and Sasser, M. 1980. Prevention - The key to controlling bacterial spot and bacterial speck of tomato. *Plant Disease* 64:831-834.

Graves, A.S., and Alexander, S.A. 2002. Managing bacterial speck and spot of tomato with acibenzolar-*S*-methyl in Virginia. online. *Plant Health Progress*. doi:10.1094/PHP-2002-0220-01-RS.

Guo, J. H., Qi, H. Y., Guo, Y. H., Ge, H. L., Gong, L. Y., Zhang, L. X., and Sun, P. H. 2004. Biocontrol of tomato wilt by plant growth-promoting rhizobacteria. *Biological Control* 29 (1):66-72.

He, Z. L. L., Yang, X. E., and Stoffella, P. J. 2005. Trace elements in agroecosystems and impacts on the environment. *Journal of Trace Elements in Medicine and Biology* 19 (2-3):125-140.

Herman, M.A.B., Nault, B.A., and Smart, C.D. 2007. Effects of plant growth-promoting rhizobacteria on bell pepper production and green peach aphid infestations in New York. *Crop Protection* In Press.

Jetiyanon, K., Fowler, W. D., and Kloepper, J. W. 2003. Broad-spectrum protection against several pathogens by PGPR mixtures under field conditions in Thailand. *Plant Disease* 87 (11):1390-1394.

Jones, J.B., Stall, R.E., and Zitter, T.A., eds. 1991. *Compendium of Tomato Diseases*. St. Paul: APS Press.

Kempster, V. N., Scott, E. S., and Davies, K. A. 2002. Evidence for systemic, cross-resistance in white clover (*Trifolium repens*) and annual medic (*Medicago truncatula* var *truncatula*) induced by biological and chemical agents. *Biocontrol Science and Technology* 12 (5):615-623.

Kennedy, I. R., Choudhury, A. T. M. A., and Kecskes, M. L. 2004. Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? *Soil Biology & Biochemistry* 36 (8):1229-1244.

Kloepper, J. W., Ryu, C. M., and Zhang, S. A. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94 (11):1259-1266.

Kokalis-Burelle, N., Vavrina, C. S., Rosskopf, E. N., and Shelby, R. A. 2002. Field evaluation of plant growth-promoting Rhizobacteria amended transplant mixes and soil solarization for tomato and pepper production in Florida. *Plant and Soil* 238 (2):257-266.

Kuc, J. 1982. Induced immunity to plant disease. *BioScience* 32 (11):854-860.

Kuc, J. 2001. Concepts and direction of induced systemic resistance in plants and its application. *European Journal of Plant Pathology* 107:7-12.

Lawton, M. B., and Macneill, B. H. 1986. Occurrence of race 1 of *Pseudomonas syringae* pv. *tomato* on field tomato in southwestern Ontario. *Canadian Journal of Plant Pathology* 8 (1):85-88.

Lorenzo, O., and Solano, R. 2005. Molecular players regulating the jasmonate signalling network. *Current Opinion in Plant Biology* 8 (5):532-540.

Louws, F. J., Wilson, M., Campbell, H. L., Cuppels, D. A., Jones, J. B., Shoemaker, P. B., Sahin, F., and Miller, S. A. 2001. Field control of bacterial spot and bacterial speck of tomato using a plant activator. *Plant Disease* 85 (5):481-488.

Madigan, M.T., Martinko, J.M., and Parker, J., eds. 2003. *Brock Biology of Microorganisms*. Tenth ed. Upper Saddle River: Pearson Education, Inc.

McSpadden Gardener, B.B., and Fravel, D.R. 2002. Biological control of plant pathogens: Research, commercialization, and application in the USA. *Plant Health Progress*.

Murphy, J. F., Reddy, M. S., Ryu, C. M., Kloepper, J. W., and Li, R. H. 2003. Rhizobacteria-mediated growth promotion of tomato leads to protection against Cucumber mosaic virus. *Phytopathology* 93 (10):1301-1307.

Nair, C. B., Anith, K. N., and Sreekumar, J. 2007. Mitigation of growth retardation effect of plant defense activator, acibenzolar-S-methyl, in

amaranthus plants by plant growth-promoting rhizobacteria. *World Journal of Microbiology & Biotechnology* 23 (8):1183-1187.

Nakkeeran, S., Dilantha Fernando, W.G., and Siddiqui, Z.A. 2005. Plant growth promoting rhizobacteria formulations and its scope in commercialization for the management of pests and diseases. In *PGPR: Biocontrol and Biofertilization*, edited by Z. A. Siddiqui. Dordrecht: Springer.

Nawrath, C., Metraux, J., and Genoud, T. 2006. Chemical signals in plant resistance: salicylic acid. In *Multigenic and Induced Systemic Resistance in Plants*, edited by S. Tuzun and E. Bent. New York: Springer Science+Business Media, Inc.

Nelson, L.M. 2004. Plant growth promoting rhizobacteria (PGPR): prospects for new inoculants. In *Plant Management Network*.

Oostendorp, M., Kunz, W., Dietrich, B., and Staub, T. 2001. Induced disease resistance in plants by chemicals. *European Journal of Plant Pathology* 107 (1):19-28.

Pedley, K. F., and Martin, G. B. 2003. Molecular basis of Pto-mediated resistance to bacterial speck disease in tomato. *Annual Review of Phytopathology* 41:215-243.

Penacortes, H., Fisahn, J., and Willmitzer, L. 1995. Signals involved in wound-induced proteinase-inhibitor-II gene-expression in tomato and potato plants. *Proceedings of the National Academy of Sciences of the United States of America* 92 (10):4106-4113.

Persello-Cartieaux, F., Nussaume, L., and Robaglia, C. 2003. Tales from the underground: molecular plant-rhizobacteria interactions. *Plant Cell and Environment* 26 (2):189-199.

Pieterse, C. M. J., Van Wees, S. C. M., Ton, J., Van Pelt, J. A., and Van Loon, L. C. 2002. Signalling in rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. *Plant Biology* 4 (5):535-544.

Pradhanang, P. M., Ji, P., Momol, M. T., Olson, S. M., Mayfield, J. L., and Jones, J. B. 2005. Application of acibenzolar-*S*-methyl enhances host resistance in tomato against *Ralstonia solanacearum*. *Plant Disease* 89 (9):989-993.

Reiners, S., and Petzoldt, C., eds. 2007. *The integrated crop and pest management guidelines for vegetables*. Ithaca: Cornell University.

Ross, A. F. 1961. Localized Acquired Resistance to Plant Virus Infection in Hypersensitive Hosts. *Virology* 14 (3):329-339.

Ross, A. F. 1961. Systemic Acquired Resistance Induced by Localized Virus Infections in Plants. *Virology* 14 (3):340-358.

Ryu, C. M., Hu, C. H., Reddy, M. S., and Kloepper, J. W. 2003. Different signaling pathways of induced resistance by rhizobacteria in *Arabidopsis thaliana* against two pathovars of *Pseudomonas syringae*. *New Phytologist* 160 (2):413-420.

Scheck, H. J., Pscheidt, J. W., and Moore, L. W. 1996. Copper and streptomycin resistance in strains of *Pseudomonas syringae* from Pacific Northwest nurseries. *Plant Disease* 80 (9):1034-1039.

Schenk, P. M., Kazan, K., Manners, J. M., Anderson, J. P., Simpson, R. S., Wilson, I. W., Somerville, S. C., and Maclean, D. J. 2003. Systemic gene expression in Arabidopsis during an incompatible interaction with Alternaria brassicicola. *Plant Physiology* 132 (2):999-1010.

Schuster, D. J. 1977. Effect of tomato cultivars on insect damage and chemical control. *Florida Entomologist* 60 (3):227-232.

Siddiqui, Z.A. 2005. PGPR: prospective biocontrol agents of plant pathogens. In *PGPR: Biocontrol and Biofertilization*, edited by Z. A. Siddiqui. Dordrecht: Springer.

Silva, V.L., and Lopes, C.A. 1995. *Pseudomonas syringae* pv. *tomato* resistant to copper in copper-sprayed tomato fields. *Fitopatologia Brasileira* 20:85-89.

Smart, C.D., Borsick, M.A., and Lange, H.W. 2005. Producing disease-free transplants. Proceedings of the Empire State Fruit and Vegetable Expo, Syracuse.

Sticher, L., MauchMani, B., and Metraux, J. P. 1997. Systemic acquired resistance. *Annual Review of Phytopathology* 35:235-270.

Stout, M. J., Brovont, R. A., and Duffey, S. S. 1998. Effect of nitrogen availability on expression of constitutive and inducible chemical defenses in tomato, Lycopersicon esculentum. *Journal of Chemical Ecology* 24 (6):945-963.

Stout, M. J., Zehnder, G. W., and Baur, M. E. 2002. Potential for the use of elicitors of plant resistance in arthropod management programs. *Archives of Insect Biochemistry and Physiology* 51 (4):222-235.

Thaler, J. S., Fidantsef, A. L., Duffey, S. S., and Bostock, R. M. 1999. Trade-offs in plant defense against pathogens and herbivores: A field demonstration of chemical elicitors of induced resistance. *Journal of Chemical Ecology* 25 (7):1597-1609.

Tornero, P., Gadea, J., Conejero, V., and Vera, P. 1997. Two *PR-1* genes from tomato are differentially regulated and reveal a novel mode of expression for a pathogenesis-related gene during the hypersensitive response and development. *Mol Plant Microbe Interact* 10 (5):624-34.

Uknes, S., Mauchmani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. 1992. Acquired-Resistance in Arabidopsis. *Plant Cell* 4 (6):645-656.

Vallad, G. E., and Goodman, R. M. 2004. Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop Science* 44 (6):1920-1934.

van Kan, J. A., Joosten, M. H., Wagemakers, C. A., van den Berg-Velthuis, G. C., and de Wit, P. J. 1992. Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum*. *Plant Molecular Biology* 20 (3):513-27.

van Loon, L. C., Bakker, P. A., and Pieterse, C. M. 1998. Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology* 36:453-83.

van Loon, L. C., Geraats, B. P. J., and Linthorst, H. J. M. 2006. Ethylene as a modulator of disease resistance in plants. *Trends in Plant Science* 11 (4):184-191.

van Wees, S. C. M., de Swart, E. A. M., van Pelt, J. A., van Loon, L. C., and Pieterse, C. M. J. 2000. Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America* 97 (15):8711-8716.

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahlgoy, P., Metraux, J. P., and Ryals, J. A. 1991.

Coordinate Gene Activity in Response to Agents That Induce Systemic Acquired-Resistance. *Plant Cell* 3 (10):1085-1094.

Wilson, M., Campbell, H. L., Ji, P., Jones, J. B., and Cuppels, D. A. 2002. Biological control of bacterial speck of tomato under field conditions at several locations in North America. *Phytopathology* 92 (12):1284-1292.

Young, Dye, D.W., and Wilkie, J.P. 1986. Bacterial Speck. In *Vegetable Diseases and Their Control*, edited by A. F. Sherf. New York: Wiley.

Zehnder, G., Kloepper, J., Tuzun, S., Yao, C. B., Wei, G., Chambliss, O., and Shelby, R. 1997. Insect feeding on cucumber mediated by rhizobacteria-induced plant resistance. *Entomologia Experimentalis Et Applicata* 83 (1):81-85.

CHAPTER TWO

INDUCTION OF PLANT DEFENSE RESPONSE PATHWAYS BY PLANT ACTIVATORS AND *PSEUDOMONAS SYRINGAE* PV. *TOMATO* IN GREENHOUSE-GROWN TOMATOES

ABSTRACT

Plant activators provide an appealing management option for bacterial diseases of greenhouse-grown tomatoes. Two types of plant activators, one that induces systemic acquired resistance (SAR) and a second that activates induced systemic resistance (ISR), were evaluated for control of *Pseudomonas syringae* pv. *tomato* and effect on plant defense gene activation. Acibenzolar-*S*-methyl (ASM, SAR-inducing compound) effectively reduced bacterial speck incidence and severity, both alone and in combination with the ISR-inducing compound. Application of ASM also led to elevated activation of the salicylic acid and ethylene signaling pathways. In contrast, the ISR-inducing compound (made up of plant growth-promoting rhizobacteria) inconsistently modified defense gene expression and did not provide disease control to the level of ASM. No negative cross-talk was observed by combining the two activators as control of bacterial speck was on par with or better than ASM alone. Implications of these findings on defense pathway interactions and greenhouse management strategies are discussed.

INTRODUCTION

Plant activators induce plant signaling pathways known as systemic acquired resistance (SAR) or induced systemic resistance (ISR) (Stout et al. 1999; Vallad and Goodman 2004). Commercially available compounds that induce SAR (such as acibenzolar-*S*-methyl) and ISR-inducing plant growth-promoting rhizobacteria (PGPR) have been shown to be effective for disease control in tomato (Anith et al. 2004; Domenech et al. 2006; Kloepper, Ryu et al. 2004; Kloepper, Reddy et al. 2004; Louws et al. 2001; Pradhanang et al. 2005). However, few studies examine utilization of these compounds together as an integrated control strategy or have investigated the impact on signaling pathways (Anith et al. 2004; Fakhouri et al. 2004; Ji et al. 2006).

Marker genes can be used to better understand how these products activate plant signaling pathways. Induction of the salicylic acid (SA) signaling pathway or systemic required resistance (SAR) can be followed using the acidic *PR-1* marker (Block et al. 2005; Tornero et al. 1997; van Kan et al. 1992). This gene is activated by compounds such as ASM and in response to abiotic and biotic stress (Durrant and Dong 2004; Friedrich et al. 1996). Ethylene (ET) is thought to enhance and stimulate defense responses; basic *PR-1* is marker for activation of this pathway (Block et al. 2005; Tornero et al. 1997; van Kan et al. 1992; van Loon et al. 2006). This signaling pathway may be involved in PGPR-plant interaction due to the role of ethylene in expression of ISR. A proteinase inhibitor, *Pin2*, is used to measure induction of the wound-inducible jasmonic acid (JA) pathway (Bowles 1998; Fidantsef et al. 1999; Penacortes et al. 1995). This pathway is activated when a plant is

wounded, such as by insect herbivory and is also thought to be involved with ISR (Pieterse et al., 2002; Kloepper et al., 2004; Bostock, 2005).

Plant defense response pathways are interconnected and antagonism and synergy between these three pathways has been reported (Bostock 2005; Lorenzo and Solano 2005). Activation of ISR and SAR simultaneously in a laboratory setting led to additive disease control though no apparent cross-talk between defense response pathways in the *P. syringae* pv. *tomato*-*Arabidopsis* pathosystem (van Wees et al. 2000).

In New York State, tomato production is predominantly for fresh market (Reiners and Petzoldt 2007). Tomato seedlings are grown in the greenhouse until five-six weeks of age then transplanted into the field. Large numbers of plants in a relatively small, confided space with overhead irrigation facilitates rapids spread of bacterial diseases through a greenhouse (Smart et al. 2005). The identification of control strategies that could effectively control bacterial disease in the greenhouse could aid transplant production.

The goal of this study was to determine whether the SAR plant activator (ASM) and the PGPR plant activator (*Bacillus* spp.), used alone or in combination, would effectively control bacterial speck in tomato. Additionally, the timing and relative increase in tomato signaling pathway gene expression between each treatment individually and both activators together were compared to untreated control plants. Implications for the use of plant activators in bacterial speck disease management strategies are discussed.

MATERIALS AND METHODS

Plant materials and treatments. Tomato (*Solanum lycopersicon*) cultivar Sunchief VF was used for all experiments. The greenhouse used for these experiments was kept at 23-26°C (day) and 20-22°C (night) with 15 hours of natural light and approximately 40% relative humidity. Tomato seeds were sown in 128 cell polystyrene flats in the greenhouse in Cornell mix (a soilless peat mixture), perlite and vermiculite 4:1:1. Tomatoes were divided into four treatments: untreated control, plants grown in the presence of PGPR, ASM applied to foliage, and the combination of plants grown in the presence of PGPR and foliage treated with ASM. The PGPR treatment, BioYield Concentrate (Bayer CropScience, Research Triangle Park, NC), was mixed evenly into the potting mix at planting (1.2 kg/m^3). This PGPR compound contains 5.0e^9 endospores per cm^3 of two bacterial strains, *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a. At four weeks, all tomatoes were transferred to four-inch pots. Six to seven-week old plants were used for this study. Acibenzolar-*S*-methyl (Actigard 50 WG, Syngenta Crop Protection, Greensboro, NC) was applied at the highest recommended rate (52 g/ha) four days prior to pathogen inoculation.

Pathogen inoculation and disease rating. The experiment was arranged in a randomized complete block design, with three replicates (or plots) of each of the four treatments at each of the four tissue collection times (see tissue collection below) per experiment. Plots consisted of three potted tomato plants, thus with three plots per treatment, there were a total of 9 plants per treatment and collection time per experiment. From each plot of three plants, one was used for disease rating, while tissue was collected from the other two

to test for defense response gene expression (see below). Each experiment consisted of 144 plants (3 plants per plot X 3 replicates (plots) X 4 collection time points X 4 treatments) and the entire experiment was repeated three times.

One hundred milliliters of *Pseudomonas syringae* pv. *tomato* (Pst) strain A9 in liquid nutrient broth (Schaad et al. 2001) was grown and shaken at 180 rpm overnight at 28ºC. Five milliliters of this inoculum was transferred to each of 15 flasks containing 500 ml of nutrient broth and again grown overnight with shaking. Prior to inoculation, bacteria were spun down at 20,000xG for 15 minutes and rinsed with 10 mM $MgCl_2$. Bacteria were resuspended in 10 mM $MgCl_2$ containing 200ul/L of the surfactant Silwet L77 (Helena Chemical, Collierville, TN) to a concentration of 10^8 CFU/ml and applied to runoff using a pump sprayer. The pathogen was applied at 7:00 PM, while all tissue collection was performed at 7:00 AM.

One plant from each treatment plot was observed seven days after inoculation and rated for disease incidence and severity. Number of infected leaflets per plant was determined and all leaflets from each plant were placed into one of six rating categories: 0, 1-10, 11-25, 26-50, 51-100, and >100 lesions per leaflet.

Analyses of disease ratings. Bacterial speck incidence, measured by the proportion of leaflets per plant containing bacterial lesions, was analyzed using quasi-likelihood models (McCullagh 1983). Means were compared using a Chi-square test at $P = 0.05$. An ordinal proportional odds logistic regression was used to model the relationship between treatment and bacterial speck disease severity (Scott et al. 1997). The odds of a treatment resulting in less

disease severity than the untreated control (having fewer numbers of leaflets in higher disease severity categories) was determined with a 95% confidence interval for each of the three experiments.

Tissue collection and RNA extraction and purification. Tomato tissue (all leaflets above the three oldest leaves were removed) was collected from two plants per group at four time points (-12, 12, 36, and 60 hours relative to pathogen inoculation, Figure 2.1). Leaf tissue from both plants was combined in a single bag, flash frozen in liquid nitrogen immediately after collection and stored at -80°C. RNA was extracted from 1 g of tissue using the SV Total RNA Isolation System (Promega Corporation, Madison, WI) and further DNase treated with Turbo DNA-*free* (Ambion Inc., Austin, TX). Samples were run on a 1.2% agarose formaldehyde gel to check for degradation.

Quantification of gene expression using qRT-PCR. Two-step real-time PCR was performed using the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) and used fluorogenic probe technology. Two ug of total RNA was used to generate cDNA with the iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. Controls lacking reverse transcriptase were included to check for DNA contamination. Gene expression was quantified from each cDNA sample using four tomato genes, actin (a housekeeping gene), acidic *PR-1*, basic *PR-1*, and *Pin2*. Real-time PCR primers and probes for tomato acidic and basic *PR-1* were identical to those described by Block et al. (Block et al. 2005). Tomato actin and *Pin2* primers and probes were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) and were as follows: actin probe 5'-/6-

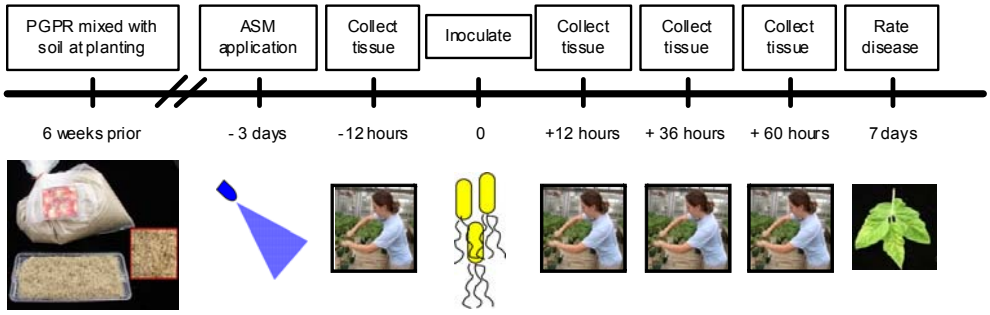


Figure 2.1. Time line of treatment application, pathogen inoculation and tissue collection. Timing is relative to inoculation with *Pseudomonas syringae* pv. *tomato*.

FAM/CGTTTGGATCTTGCTGGTCGTGATTTAACT/TAMRA/-3'; actin forward primer 5'-TTGCCGCATGCCATTCT-3'; actin reverse primer 5'-TCGGTGAGGATATTCATCAGGTT-3'; *Pin2* probe 5'-/6-FAM/TGTGGTAATCTTGGGTTCGGGATATGCC/TAMRA/-3'; *Pin2* forward primer 5'-TGATGCCAAGGCTTGTACTAGAGA-3'; *Pin2* reverse primer 5'-AGCGGACTTCCTTCTGAACGT-3' (IntergratedDNATechnologies, Coralville, IA).

Real-time quantitative PCR reactions were carried out in triplicate; each reaction used the iQ Supermix Kit (Bio-Rad Laboratories) and consisted of 1X Mastermix, forward and reverse primers (300 nM final concentration) and 200 nM fluorogenic probe. Reaction parameters consisted of 95°C for 4 min, followed by 45 cycles of 95°C for 10 sec, 50°C for 1 min, and 72°C for 30 sec. In addition to cDNA reaction samples lacking reverse transcriptase, reactions with no cDNA template were also included as negative controls.

Initial transcript levels were determined using the standard curve method (User Bulletin #2, ABI PRISM 7700 Sequence Detection System, Relative quantification of gene expression, 2001, Applied Biosystems). Serial dilutions of tomato total genomic DNA were used to generate standard curves (Yun et al. 2006). Standard curve construction was accomplished by plotting the threshold cycle (Ct) against the logarithm of the known tomato DNA dilutions. The absolute quantity of the product in each sample was calculated from these curves (Ding et al. 2004; Mittapalli et al. 2006). Subsequently, tomato actin (used as an internal control) was used to normalize tomato defense gene expression and generate relative expression values (REVs) (Beaubois et al. 2007). Relative expression change was calculated by

calibrating samples to the mean REV of the three replicates (within each experiment) prior to pathogen inoculation (-12 hour time point) for each of the four treatments. The log of the REVs for each of the three marker genes tested was used to determine statistical significance. An ANOVA (Analysis of Variance) using the Proc Mixed procedure and SAS software version 9.1(Cary, NC, USA) was used to analyze these values (Mittapalli et al. 2006). Differences between treatments were evaluated using orthogonal contrasts at each time point and *P*-values <0.05 were considered statistically significant (Mittapalli et al. 2006).

RESULTS

Bacterial speck incidence. Incidence of *Pseudomonas syringae* pv. *tomato,* as measured by the mean proportion of infected leaflets per plant, followed a similar pattern in all three experimental replications (*P*=0.30, Table 2.1). Incidence was not significantly different between the untreated control and PGPR-treated plants (*P*=0.119, *P*=0.9177, *P*=0.12, respectively). Both ASM (*P*<0.0001, *P*=0.0244, *P*<0.0001, respectively) and ASM in combination with the PGPR compound (*P*<0.0001, *P*=0.0003, *P*<0.0001) significantly reduced bacterial speck incidence in all three experimental replicates.

Bacterial speck severity. In the three experiments, disease severity in all treatments differed significantly from the untreated control (*P*<0.0001), with the PGPR differing the least and both ASM treatments having similar differences from the control (Tables 2.2 & 2.3). The combined total number of leaflets (from all three experiments) in each disease severity category is depicted by treatment in Table 2.2. The odds of treatment plants having less severe disease than the untreated control are broken down by experiment in Table

Table 2.1. Bacterial speck disease incidence over three greenhouse experiments.

Treatment	Bacterial Speck Incidence							
	Untreated Control		PGPR		ASM		ASM + PGPR	
	Proportion[a]	Average Number[b]	Proportion	Average Number	Proportion	Average Number	Proportion	Average Number
Experiment 1[c]	0.53 +/- 0.04 a	16/31	0.44 +/- 0.04 a	15/33	0.18 +/- 0.03 b	6/32	0.15 +/- 0.03 b	5/33
Experiment 2	0.42 +/- 0.05 a	23/55	0.42 +/- 0.05 a	22/52	0.26 +/- 0.05 b	14/55	0.16 +/- 0.04 b	9/58
Experiment 3	0.46 +/- 0.04 a	25/54	0.37 +/- 0.04 a	19/51	0.15 +/- 0.03 b	9/57	0.15 +/- 0.03 b	9/56

[a]Mean proportion of infected leaflets per plant averaged for three replicates +/- standard error.

[b]Mean number of infected leaflets over the mean number of leaflets per plant.

[c]Means within a row sharing the same letter are not statistically different.

Table 2.2. Bacterial speck severity.

	Bacterial speck severity			
Severity[a]	Untreated Control[b]	PGPR	ASM	ASM + PGPR
1-10	593	568	306	266
11-25	113	67	30	11
26-50	46	26	6	0
51-100	14	2	0	0
>100	5	2	0	0

[a]Disease severity classes signifying the number of lesions per leaflet.

[b]Number of leaflets in each severity class are the combined total for all three experiments.

Table 2.3. Odds of treated plants having lower disease severity than untreated control.

	PGPR[a]		ASM		ASM + PGPR	
	Odds ratio	95% CI	Odds ratio	95% CI	Odds ratio	95% CI
Experiment 1	1.58	1.20 - 2.08	5.76	4.14 - 8.01	6.54	4.68 - 9.13
Experiment 2	1.04	0.83 - 1.29	2.08	1.66 - 2.62	4.08	3.16 - 5.27
Experiment 3	1.53	1.23 - 1.91	5.13	3.96 - 6.65	5.04	3.89 - 6.52

[a]For each experiment, the odds ratio estimates the increased likelihood of treated plants (PGPR, ASM or ASM+PGPR) having lower disease severity (fewer leaflets in the higher severity classes) than the untreated control. Odds ratios are given with 95% confidence levels. Disease severity did not differ significantly from the untreated control (an odds ratio of 1.0) if the confidence intervals contain the value 1.0.

2.3. Plants grown in the presence of the PGPR compound alone were 1.58, 1.04 or 1.53 times more likely than the control to have fewer numbers of leaflets in higher disease severity categories. Both ASM treatments provided disease control as plants exhibited fewer, severely infected leaflets. Treatment with ASM produced plants that were 5.80, 2.08 or 5.13 fold more likely to be less severely infected than the untreated control. The combination of ASM and the PGPR compound produced similar results to the ASM compound alone, with treated plants 6.54, 4.08 or 5.04 times more likely to have lower disease severity than the untreated control for each of the three experiments, respectively.

Treatment and signaling pathway activation. Level of defense gene expression was effectively quantified for the three signaling pathway marker genes (relative to actin) via qRT-PCR for each of the four treatments. Complete results for each of the three experiments are divided by signaling pathway (Figs. 2.2-2.4) and are presented by treatment (untreated control, PGPR-treated, ASM-treated, ASM+PGPR-treated) below.

*Response of untreated plants***.** In all three experiments, the untreated control demonstrated low levels of acidic *PR-1* expression prior to pathogen inoculation (Figure 2.2, maroon bars). Over the course of the experiment, activation of the salicylic signaling pathway increased gradually to highest levels at sixty hours after inoculation (16.9, 10.2, 4.9 fold increase from -12 hours, P=0.0005, P<0.0001, P<0.0001).

Basic *PR-1* expression followed a similar pattern in untreated control plants as acidic *PR-1*, with low expression levels prior to pathogen inoculation, increasing to highest levels 60 hours after inoculation (Figure 2.3, maroon

Figure 2.2. Expression pattern of the salicylic acid pathway in relationship to inoculation with *Pseudomonas syringae* pv. *tomato*. Top (A), middle (B) and bottom (C) graphs represent acidic *PR-1* induction patterns in experiments 1, 2 and 3, respectively. Maroon bars represent mean expression level of three replicated plots of untreated control plants, green bars signify PGPR-treated plants, orange bars indicate ASM-treated plants, and yellow bars correspond to plants treated with both ASM and PGPR. Bars represent average induction (+/- SE) of gene transcripts normalized to the housekeeping gene actin for three replicate plots. Gene activation was then normalized to the mean expression value prior to bacterial inoculation (-12 hours) for each treatment group. Plants were inoculated with *P. syringae* pv. *tomato* at time zero.

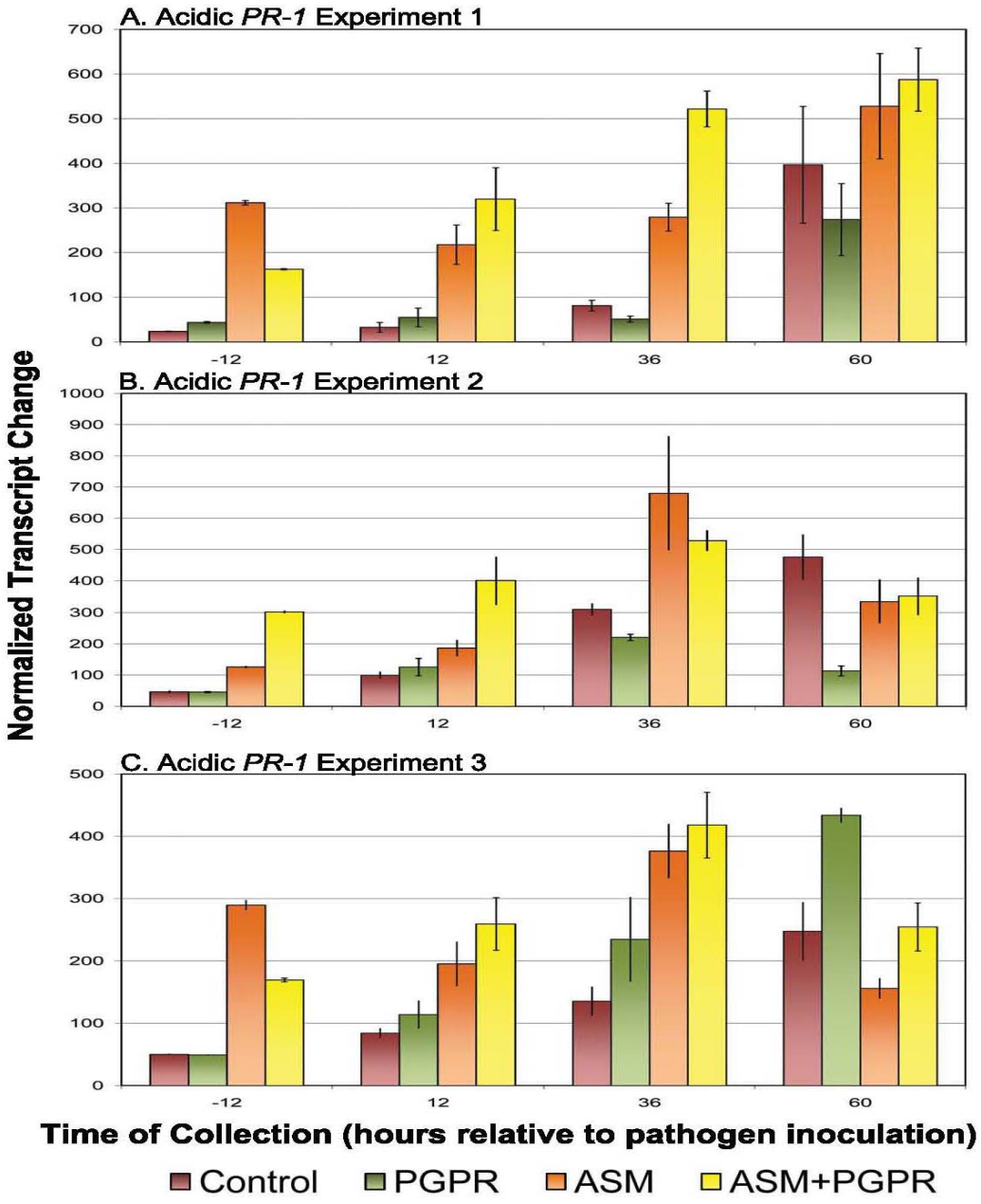
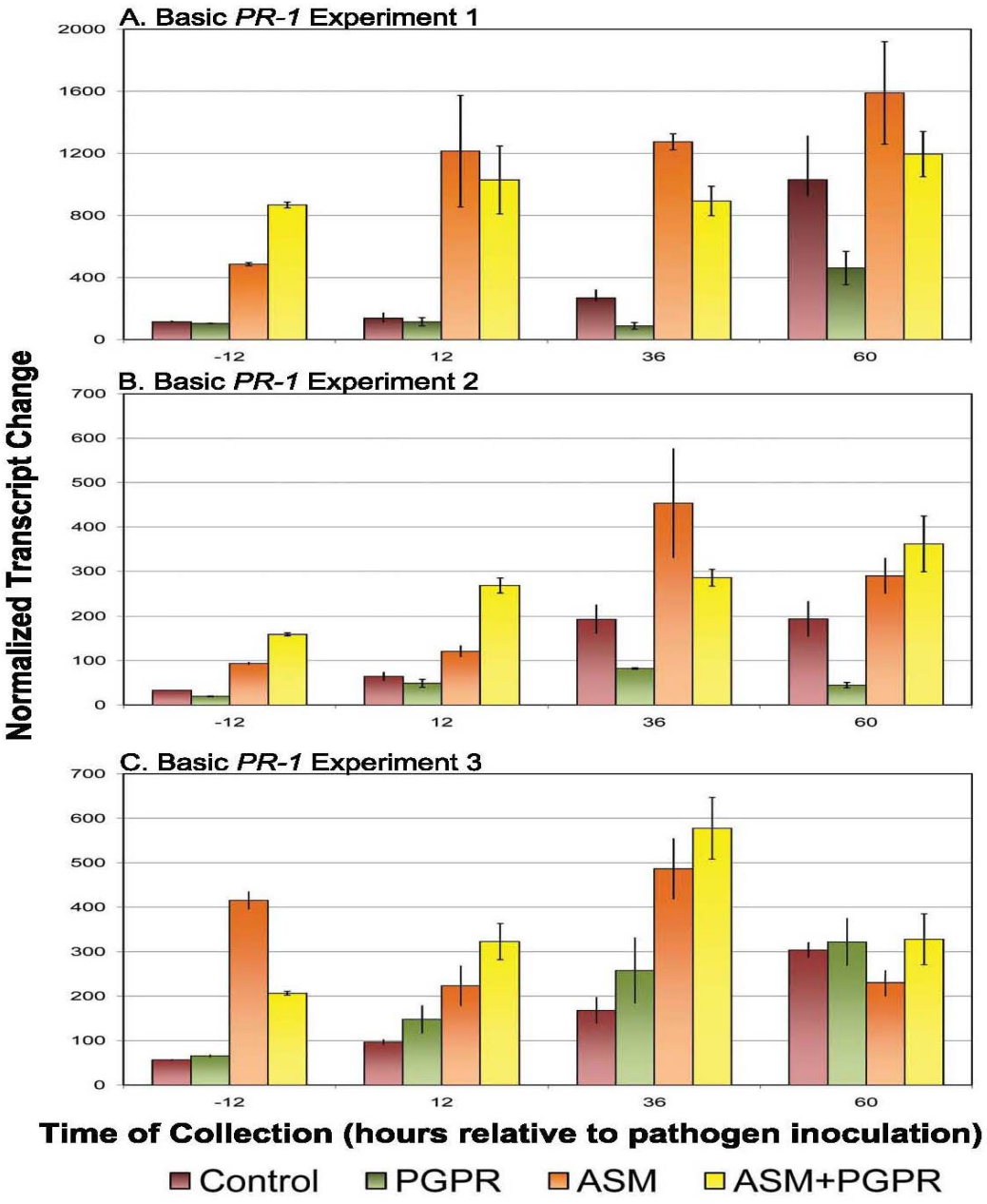

A. Acidic PR-1 Experiment 1
B. Acidic PR-1 Experiment 2
C. Acidic PR-1 Experiment 3
Normalized Transcript Change
Time of Collection (hours relative to pathogen inoculation)
-12
12
36
60
Control
PGPR
ASM
ASM+PGPR

Figure 2.3. Expression pattern of the ethylene pathway in relationship to inoculation with *Pseudomonas syringae* pv. *tomato*. Top (A), middle (B) and bottom (C) graphs represent induction patterns of basic *PR-1*, in experiments 1, 2 and 3, respectively. Maroon bars represent mean expression level of three replicated plots of untreated control plants, green bars signify PGPR-treated plants, orange bars indicate ASM-treated plants, and yellow bars correspond to plants treated with both ASM and PGPR. Bars represent average induction (+/- SE) of gene transcripts normalized to the housekeeping gene actin for three replicate plots. Gene activation was then normalized to the mean expression value prior to bacterial inoculation (-12 hours) for each treatment group. Plants were inoculated with *P. syringae* pv. *tomato* at time zero.


A. Basic PR-1 Experiment 1
B. Basic PR-1 Experiment 2
C. Basic PR-1 Experiment 3
Normalized Transcript Change
2000
1600
1200
800
400
0
700
600
500
400
300
200
100
-12
12
36
60
Time of Collection (hours relative to pathogen inoculation)
Control
PGPR
ASM
ASM+PGPR

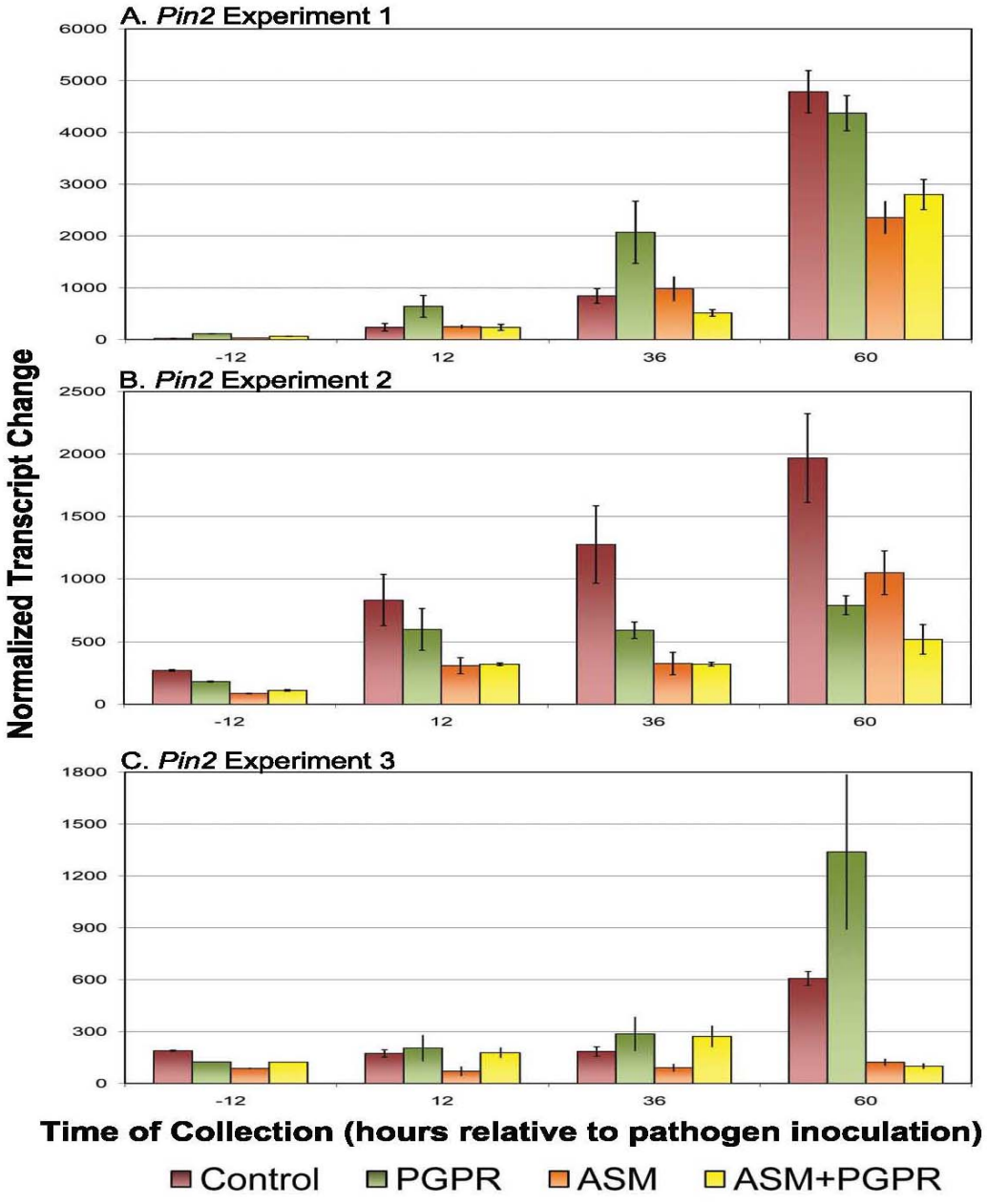
bars). By sixty hours, basic PR-1 expression was significantly higher than levels observed prior to inoculations in each of the three experiments (8.9, 5.8, 5.4 fold increase from -12 hours, P=0.0005, P=0.0001, P<0.0001).

Wound-induced signaling in untreated control plants dramatically increased from low levels 12 hours prior to inoculation to highest levels at 60 hours (Figure 2.4, maroon bars). Large fold changes were observed in the jasmonic acid signaling pathway in each experiment (205.7, 7.2, 3.2 fold change from uninoculated plants at -12 hours, P<0.0001, P<0.0001, P<0.0001).

Response of PGPR-treated plants. In all three experiments, PGPR-treated plants responded in a similar fashion to the untreated control, with low levels of acidic *PR-1* expression prior to pathogen inoculation (Figure 2.2, green bars). Induction of the salicylic acid signaling pathway increased to highest expression levels at 60 (Figure 2.2 A, C) and 36 (Figure 2.2 B) hours post infection (6.3, 4.8, 8.8 fold increase from -12 hours, P=0.0005, P<0.0001, P=0.0002). Peak expression relative to the untreated control was varied between the three experiments; no significant differences were observed in experiment one, PGPR-treated plants had lower acidic *PR-1* activation in experiment two and higher in experiment three.

Activation of the ethylene signaling pathway was similar to acidic *PR-1*, with low expression levels prior to inoculation and increasing to highest levels 36 (Figure 2.3 B) or 60 (Figure 2.3 A, C) hours after inoculation. Basic *PR-1* expression was significantly higher than levels observed prior to inoculations in each of the three experiments (4.4, 4.2, 4.9 fold increase from -12 hours, P=0.0155, P<0.0001, P<0.0009). In two experiments, PGPR-treated plants

Figure 2.4. Expression pattern of the jasmonic acid pathway in relationship to inoculation with *Pseudomonas syringae* pv. *tomato*. Top (A), middle (B) and bottom (C) graphs represent induction patterns of *Pin2* in experiments 1, 2 and 3, respectively. Maroon bars represent mean expression level of three replicated plots of untreated control plants, green bars signify PGPR-treated plants, orange bars indicate ASM-treated plants, and yellow bars correspond to plants treated with both ASM and PGPR. Bars represent average induction (+/- SE) of gene transcripts normalized to the housekeeping gene actin for three replicate plots. Gene activation was then normalized to the mean expression value prior to bacterial inoculation (-12 hours) for each treatment group. Plants were inoculated with *P. syringae* pv. *tomato* at time zero.


A. Pin2 Experiment 1
B. Pin2 Experiment 2
C. Pin2 Experiment 3
Normalized Transcript Change
Time of Collection (hours relative to pathogen inoculation)
-12
12
36
60
Control
PGPR
ASM
ASM+PGPR

exhibited significantly lower activation of the ethylene signaling pathway (Figure 2.3 A, B) while no significant differences were found in the third experiment (Figure 2.3 C).

Similar to untreated control plants, wound-induced signaling dramatically increased from low levels 12 hours prior to inoculation to highest levels at 60 hours in PGPR-treated plants (Figure 2.4, green bars). At peak induction of the jasmonic acid signaling pathway, large fold changes were observed in each experiment (39.0, 4.3, 10.7 fold increase from -12 hours, P=0.0001, P<0.0093, P<0.0049).

Response of ASM-treated plants. Treatment with ASM activated acidic *PR-1* prior to inoculation to levels 3-13 fold higher than the untreated control (Figure 2.2, orange bars). Levels remained elevated throughout the course of the experiment, increasing after inoculation to highest levels at the 36 (Figure 2.2 B & C) or 60 hour time points (Figure 2.2 A).

Acibenzolar-*S*-methyl treatment also induced the ethylene signaling pathway prior to pathogen inoculation to levels 3-7 fold higher than the untreated control (Figure 2.3, orange bars). Peak activation followed a similar pattern to acidic *PR-1* expression in all three experiments, with levels increasing after inoculation to highest levels at 36 (Figure 2.3 B, C) or 60 (Figure 2.3 A) hours post inoculation.

Over the time course of the experiment, *Pin2* was upregulated in ASM-treated plants, though expression levels observed were 50-80 % lower than untreated controls (Figure 2.4, orange bars). Induction of the jasmonic acid signaling pathway increased to highest levels by the 60 hour time point in each

experiment (74.1, 12.1, 1.4 fold change from uninoculated plants at -12 hours, $P<0.0001$, $P<0.0001$, $P=0.2239$).

Response of ASM and PGPR-treated plants. As seen with ASM treatment alone, the combination of plant activators induced acidic *PR-1* activation 3-7 fold higher than the untreated control prior to inoculation (Figure 2.2, yellow bars). Levels increased over the course of the experiment to peak levels at the 36 (Figure 2.2 B & C) or 60 hour time points (Figure 2.2 A).

The ethylene signaling pathway was also induced in plants treated with ASM and PGPR prior to inoculation (4-7.5 fold higher than the untreated control) (Figure 2.3, yellow bars). A similar pattern to acidic *PR-1* expression was observed following inoculation, with levels increasing to highest levels at 36 (Figure 2.3 C) or 60 (Figure 2.3 A, B) hours. One difference from acidic *PR-1* expression was observed in experiment 2; peak activation of the ethylene signaling pathway occurred one day later, at the 60 hour time point (Figs. 2.2 B, 2.3 B).

Expression of *Pin2* increased following inoculation in ASM and PGPR-treated plants, with significantly lower expression levels (41 - 84 % lower) than untreated controls (Figure 2.4, yellow bars). Greatest induction of the jasmonic acid signaling pathway occurred at the 60 hour (Figure 2.4 A & B) or 36 (Figure 2.4 C) time point (44.4, 4.7, 2.2 fold change from uninoculated plants at -12 hours, $P<0.0001$, $P<0.0001$, $P=0.6371$).

DISCUSSION

Data presented in this study provide support for the incorporation of plant activators in greenhouse management of bacterial diseases. Combination of two plant activators could benefit tomato transplant production and protect from bacterial speck.

Treatment with the PGPR compound reduced bacterial speck symptoms relative to the untreated control in two of the three experiments though not to a commercially acceptable level (Tables 2.1 – 2.3). In contrast, ASM effectively reduced bacterial speck incidence and severity both alone and in combination with the PGPR compound. Use of both activators together resulted in lower disease incidence than ASM alone in one experiment and lower infection severity in two experiments.

Disease results correlated to wound-induced gene expression (*Pin2*). Plants exhibiting higher levels of disease (untreated control, PGPR-treated) demonstrated a greater response of the jasmonic acid signaling pathway to infection (Figure 2.4). Previous studies have found production of coronatine, a jasmonic acid mimic and phytotoxin, by *P. syringae* pv. *tomato* to be correlated with induction of wound-responsive genes in susceptible tomato (Strassner et al. 2002; Zhao et al. 2003). Alternatively, ASM-treated plants displayed significantly lower levels of wound-induced signaling, alone or in combination with the PGPR compound, than the untreated control at 60 hours. This reflects the reduced disease severity in plants treated with ASM. Response of PGPR-treated plants did not reflect level of disease relative to the untreated control, as response ranged from the same, lower, and higher *Pin2* expression in PGPR plots. Part of this variation may be due to high internal variability in

the PGPR-treated plants in experiment 3 at the 60 hour time point. One replicate demonstrated much higher *Pin2* expression than the other two, consequently inflating the mean expression and standard error. While gene expression differences between the three experiments may also be due to environmental differences in the greenhouse that influenced the growth of the pathogen, the outcome was still the same. Treatment with ASM effectively controlled disease, whereas the PGPR compound did not provide commercially acceptable control. Utilization of both compounds in concert did not negatively impact SAR signaling as disease control was equivalent to the ASM compound alone.

Previous studies have found SA and ET levels to increase in compatible interactions between bacterial pathogens and tomato (Block et al. 2005; Zhao et al. 2003). Plants that exhibited susceptibility to bacterial speck (untreated control and PGPR-treated) also demonstrated increasing SA and ET levels over the course of the experiment following inoculation. Untreated controls reached peak expression of both acidic and basic *PR-1* at 60 hours after inoculation, where as the response of PGPR-treated plants was more variable.

Treatment with ASM induced the salicylic acid and ethylene signaling pathways prior to inoculation, alone or in combination with the PGPR compound, in all three experiments. Plants remained activated throughout the duration of the experiment and each pathway was induced to a greater degree following pathogen inoculation. Degree of activation varied between the three experiments. In ASM-treated plants, salicylic acid expression was higher in exp. 1, levels were similar in exp. 2, and ethylene expression was higher in

exp. 3. In contrast, ASM+PGPR-treated plots exhibited higher ethylene expression in exp 1, salicylic acid expression was higher in exp 2, and similar expression levels were observed in exp. 3. Levels of acidic and basic *PR-1* expression also fluctuated between ASM and ASM+PGPR-treated plants; hence there was no discernable pattern of whether one treatment had a greater effect on salicylic acid and ethylene signaling pathways.

In previous studies, PGPR compounds have not been associated with major changes in PR gene expression (Van Loon 1999) though some strains of *Bacillus* have been found to activate defense-related pathways (Ongena et al. 2005). It is possible that the PGPR compound primed plants to respond more quickly and to a greater degree following infection (Fig 2.2 C, 2.3 C, 2.4 A, C) though the effect was inconsistent and did not provide sufficient disease control. There was no evidence for antagonistic effects of activating the SAR and ISR pathways in concert as no consistent differences were found in expression of the three marker genes between ASM and ASM + PGPR-treated plants. Synergy between the two pathways is possible as disease incidence and severity was reduced in some of the experiments, though not significantly.

Although the PGPR compound used in this study failed to provide sufficient bacterial speck control, growth-promoting benefits of this product are were not analyzed. Previous work has shown that PGPR can be effective in transplant and field production (Kloepper, Ryu, and Zhang 2004; Kloepper, Reddy et al. 2004). While copper is currently the most widely used control measure, incorporation of ASM into greenhouse transplant production could be useful for bacterial speck control. Combining the two plant activators may

provide the disease control benefits of ASM while boosting plant yield, however further investigation of these plant activators in a production system is necessary.

REFERENCES

Anith, K. N., Momol, M. T., Kloepper, J. W., Marois, J. J., Olson, S. M., and Jones, J. B. 2004. Efficacy of plant growth-promoting rhizobacteria, acibenzolar-*S*-methyl, and soil amendment for integrated management of bacterial wilt on tomato. *Plant Disease* 88 (6):669-673.

Beaubois, E., Girard, S., Lallechere, S., Davies, E., Paladian, F., Bonnet, P., Ledoigt, G., and Vian, A. 2007. Intercellular communication in plants: Evidence for two rapidly transmitted systemic signals generated in response to electromagnetic field stimulation in tomato. *Plant Cell and Environment* 30 (7):834-844.

Block, A., Schmelz, E., O'Donnell P, J., Jones, J. B., and Klee, H. J. 2005. Systemic acquired tolerance to virulent bacterial pathogens in tomato. *Plant Physiology* 138 (3):1481-1490.

Bostock, R. M. 2005. Signal crosstalk and induced resistance: straddling the line between cost and benefit. *Annual Review of Phytopathology* 43:545-580.

Bowles, D. 1998. Signal transduction in the wound response of tomato plants. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 353 (1374):1495-1510.

Ding, J. Y., Jia, J. W., Yang, L. T., Wen, H. B., Zhang, C. M., Liu, W. X., and Zhang, D. B. 2004. Validation of a rice specific gene, sucrose phosphate synthase, used as the endogenous reference gene for qualitative and real-time quantitative PCR detection of transgenes. *Journal of Agricultural and Food Chemistry* 52 (11):3372-3377.

Domenech, J., Reddy, M. S., Kloepper, J. W., Ramos, B., and Gutierrez-Manero, J. 2006. Combined application of the biological product LS213 with *Bacillus*, *Pseudomonas* or *Chryseobacterium* for growth promotion and biological control of soil-borne diseases in pepper and tomato. *Biocontrol* 51 (2):245-258.

Durrant, W. E., and Dong, X. 2004. Systemic acquired resistance. *Annual Review of Phytopathology* 42:185-209.

Fakhouri, W. D., Neemann, M., Walker, F., and Buchenauer, H. 2004. Application of fluorescent pseudomonads in combination with acibenzolar-S-methyl induces disease resistance in tomato and tobacco. *Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection* 111 (5):494-505.

Fidantsef, A. L., Stout, M. J., Thaler, J. S., Duffey, S. S., and Bostock, R. M. 1999. Signal interactions in pathogen and insect attack: expression of

lipoxygenase, proteinase inhibitor II, and pathogenesis-related protein P4 in the tomato, *Lycopersicon esculentum*. *Physiological and Molecular Plant Pathology* 54 (3-4):97-114.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M. G., Meier, B., Dincher, S., Staub, T., Uknes, S., Metraux, J. P., Kessmann, H., and Ryals, J. 1996. A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant Journal* 10 (1):61-70.

Ji, P., Campbell, H. L., Kloepper, J. W., Jones, J. B., Suslow, T. V., and Wilson, M. 2006. Integrated biological control of bacterial speck and spot of tomato under field conditions using foliar biological control agents and plant growth-promoting rhizobacteria. *Biological Control* 36 (3):358-367.

Kloepper, J. W., Ryu, C. M., and Zhang, S. A. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94 (11):1259-1266.

Kloepper, J.W., Reddy, M.S., Rodriguez-Kabana, R., Kenney, D.S., Kokalis-Burelle, N., and Martinez-Ochoa, N. 2004. Application for rhizobacteria in transplant production and yield enhancement. *Acta Horticulturae* 631:217-229.

Lorenzo, O., and Solano, R. 2005. Molecular players regulating the jasmonate signalling network. *Current Opinion in Plant Biology* 8 (5):532-540.

Louws, F. J., Wilson, M., Campbell, H. L., Cuppels, D. A., Jones, J. B., Shoemaker, P. B., Sahin, F., and Miller, S. A. 2001. Field control of bacterial spot and bacterial speck of tomato using a plant activator. *Plant Disease* 85 (5):481-488.

McCullagh, P. 1983. Quasi-likelihood functions. *Annals of Statistics* 11 (1):59-67.

Mittapalli, O., Shukle, R.H., Sardesai, N., Giovanini, M.P., and Williams, C.E. 2006. Expression patterns of antibacterial genes in the Hessian fly. *Journal of Insect Physiology* 52:1143-1152.

Ongena, M., Duby, F., Jourdan, E., Beaudry, T., Jadin, V., Dommes, J., and Thonart, P. 2005. Bacillus subtilis M4 decreases plant susceptibility towards fungal pathogens by increasing host resistance associated with differential gene expression. *Applied Microbiology and Biotechnology* 67 (5):692-698.

Penacortes, H., Fisahn, J., and Willmitzer, L. 1995. Signals involved in wound-induced proteinase-inhibitor-II gene-expression in tomato and potato

plants. *Proceedings of the National Academy of Sciences of the United States of America* 92 (10):4106-4113.

Pradhanang, P. M., Ji, P., Momol, M. T., Olson, S. M., Mayfield, J. L., and Jones, J. B. 2005. Application of acibenzolar-*S*-methyl enhances host resistance in tomato against *Ralstonia solanacearum*. *Plant Disease* 89 (9):989-993.

Reiners, S., and Petzoldt, C., eds. 2007. *The integrated crop and pest management guidelines for vegetables*. Ithaca: Cornell University.

Schaad, N.W., Jones, J.B., and Chun, W., eds. 2001. *Laboratory guide for identification of plant pathogenic bacteria*. Third ed. St. Paul: APS Press.

Scott, S.C., Goldberg, M.S., and Mayo, N.E. 1997. Statistical assessment of ordinal outcomes in comparative studies. *Journal of Clinical Epidemiology* 50 (1):45-55.

Smart, C.D., Borsick, M.A., and Lange, H.W. 2005. Producing disease-free transplants. Proceedings of the Empire State Fruit and Vegetable Expo, Syracuse.

Stout, M. J., Fidantsef, A. L., Duffey, S. S., and Bostock, R. M. 1999. Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, Lycopersicon esculentum. *Physiological and Molecular Plant Pathology* 54 (3-4):115-130.

Strassner, J., Schaller, F., Frick, U. B., Howe, G. A., Weiler, E. W., Amrhein, N., Macheroux, P., and Schaller, A. 2002. Characterization and cDNA-microarray expression analysis of 12-oxophytodienoate reductases reveals differential roles for octadecanoid biosynthesis in the local versus the systemic wound response. *Plant Journal* 32 (4):585-601.

Tornero, P., Gadea, J., Conejero, V., and Vera, P. 1997. Two *PR-1* genes from tomato are differentially regulated and reveal a novel mode of expression for a pathogenesis-related gene during the hypersensitive response and development. *Mol Plant Microbe Interact* 10 (5):624-34.

Vallad, G. E., and Goodman, R. M. 2004. Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop Science* 44 (6):1920-1934.

van Kan, J. A., Joosten, M. H., Wagemakers, C. A., van den Berg-Velthuis, G. C., and de Wit, P. J. 1992. Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced

by virulent and avirulent races of *Cladosporium fulvum*. *Plant Molecular Biology* 20 (3):513-27.

Van Loon, L. C. 1999. Occurrence and Properties of Plant Pathogenesis-Related Proteins. In *Pathogenesis-Related Proteins in Plants*, edited by S. K. Datta and S. Muthukrishnan. Boca Raton: CRC Press.

van Loon, L. C., Rep, M., and Pieterse, C. M. J. 2006. Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology* 44:135-162.

van Wees, S. C. M., de Swart, E. A. M., van Pelt, J. A., van Loon, L. C., and Pieterse, C. M. J. 2000. Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America* 97 (15):8711-8716.

Yun, J. J., Heisler, L. E., Hwang, I. I. L., Wilkins, O., Lau, S. K., Hyrcza, M., Jayabalasingham, B., Jin, J., McLaurin, J., Tsao, M. S., and Der, S. D. 2006. Genomic DNA functions as a universal external standard in quantitative real-time PCR. *Nucleic Acids Research* 34 (12):E85-E85.

Zhao, Y., Thilmony, R., Bender, C. L., Schaller, A., He, S. Y., and Howe, G. A. 2003. Virulence systems of Pseudomonas syringae pv. tomato promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. *Plant J* 36 (4):485-99.

CHAPTER THREE

DEFENSE GENE EXPRESSION PATTERNS OF THREE SAR-INDUCED TOMATO CULTIVARS IN THE FIELD

ABSTRACT

Plant activation is an attractive disease management tool, avoiding some of the challenges of traditional chemical control by not directly impacting the pathogen. This study examined effects of acibenzolar-*S*-methyl (ASM), a plant activator that induces systemic acquired resistance, on defense response activation in three field-grown tomato cultivars in New York. Salicylic acid, ethylene and jasmonic acid-mediated responses were monitored by following expression of a marker gene for each signaling pathway using quantitative real-time PCR over the course of two ASM applications. ASM induced the salicylic acid and ethylene, but not jasmonic acid-regulated gene expression in all cultivars tested. All three cultivars demonstrated a significantly stronger gene expression response relative to the untreated control following the second ASM application. Implications of these findings on management practices are discussed.

INTRODUCTION

Plant activators, compounds that control disease without directly impacting the pathogen, could be useful tools for crop protection. Acibenzolar-*S*-methyl (ASM) is a commercially available activator derived from benzo[1,2,3]thiadiazole (BTH). It is a functional analog of salicylic acid known to stimulate the production of plant defense-related compounds and induce systemic acquired resistance (SAR) (Fidantsef et al. 1999; Kuc 2001; Thaler et al. 1999). SAR is distinguished from other plant defense responses by local and systemic activation of specific pathogenesis-related genes (PR genes) (Durrant and Dong 2004).

ASM activates defense responses in a wide range of plants, potentially providing broad-spectrum protection (Oostendorp et al. 2001). Within two hours of application, ASM can be detected in tomato leaves (both treated and distant from the application site) (Scarponi et al. 2001). Under growth chamber conditions, ASM concentration was highest two hours after treatment and levels decreased to baseline levels by forty-eight hours in distal and seventy-two hours in proximal leaves (Scarponi, Buonaurio, and Martinetti 2001). Other studies have found raised expression levels of the tomato gene P4 (a marker for SAR) four days after BTH application in the greenhouse (Fidantsef et al. 1999). One previous study examined the molecular effects of plant activators in the field and found that BTH-treated tomatoes exhibited higher, though not significantly different from the untreated control, levels of P4 mRNA expression five days after application (Thaler et al. 1999).

While ASM has been found effective against a wide range of bacterial pathogens of tomato (Anith et al. 2004; Baysal et al. 2003; Graves and

Alexander 2002; Louws et al. 2001; Pradhanang et al. 2005; Wilson et al. 2002), little is known about the influence of genotype (plant cultivar), environment and crop nutrition on induced responses. Greater understanding of the possible fitness costs and differences in activation associated with induced resistance, both in the presence and absence of pathogens, is necessary to effectively implement plant activators in a pest management program (Walters and Boyle 2005). The efficacy of ASM to control bacterial diseases of tomato has been assessed in several tomato growing areas in North America, though differences in defense gene activation between cultivars have not been compared (Louws et al. 2001).

The goal of this study was to determine if plant signaling pathways were induced at the same rate and to the same degree in three tomato cultivars under field conditions in New York. Salicylic acid, ethylene and jasmonic acid-mediated defenses were followed using marker genes and expression quantified with quantitative real-time PCR (qRT-PCR). This information is crucial to determine the optimal application regime which will enable the highest level of disease control.

MATERIALS AND METHODS

Plant Materials and Treatments. Tomato (*Solanum lycopersicum*) cultivars Rutgers, Rio Grande, and Supersonic were used in this experiment. All three cultivars exhibit resistance to Fusarium wilt and Verticillium wilt. Rutgers and Rio Grande are determinate cultivars (both widely used in research studies) whereas Supersonic is indeterminate. Tomato seeds were sown in 24 cell polystyrene flats in Cornell mix, a soil-less peat mixture, with perlite and vermiculite (4:1:1). Fertilizer containing nitrogen, phosphorus and potassium

(10-5-10) was added at a rate of 2.67 kg per cubic meter of mix. Plants were grown under natural sunlight in the greenhouse with temperatures of 23-26°C (day) and 20-22°C (night). The photoperiod in Geneva, NY, is approximately 15 hours per day from mid-May to mid-June. One week prior to field planting, seedlings were moved to an outdoor coldframe.

Field evaluation. Six-week old tomato plants were planted in early June at 45.7 cm intervals. Plants were fertilized with liquid fertilizer (15:30:15 N-P-K) prior to field planting. Each plot consisted of two plants, with two plants (~140 cm) separating each treatment (ASM-treated and the untreated control) and three replications per cultivar. Tomato fertilization, irrigation and weed control were implemented following typical production practices for NY (Reiners and Petzoldt 2007). Acibenzolar-*S*-methyl (Actigard, Syngenta Crop Protection, Greensboro, NC) was applied twice, on 29 July and 5 August at the highest labeled rate (52 g per ha).

Tissue collection and RNA extraction. Two leaves (each with five to seven leaflets) were collected from individual field plots for each of three replicates. All tissue collections were made at 7:00 AM. Tomato tissue was collected at four time points relative to each ASM application, immediately prior to the first treatment (designated as day zero) and one, two, and three days following. Tissue collection preceding the second ASM treatment occurred on day seven, with successive samples taken over the next three days (days eight, nine and ten). Leaf tissue was flash frozen in liquid nitrogen in the field immediately following collection and stored at -80°C. Total tomato RNA was isolated from leaf tissue using the hot phenol protocol (Perry and Francki 1992) as modified by Gu and colleagues (Gu et al. 2000) and further DNase

treated (Turbo DNA-*free*, Ambion Inc., Austin, TX). RNA (1 ug) was separated electrophoretically on a 1.2% agarose formaldehyde gel to check for degradation.

Quantification of gene expression using quantitative real-time PCR (qRT-PCR). Primers and fluorogenic probes for *Pin2* and actin were developed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) (Table 1). Acidic and basic *PR-1* primer and probe combinations were identical to those described by Block et al. (Block et al. 2005). All primers and probes used in this study are listed in Table 3.1.

Two-step real-time quantitative PCR (qRT-PCR) was performed using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The first step consisted of generating cDNA from total RNA (2 µg) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Each reaction (40 µL) received 1X iScript reaction mix (RNAse inhibitors, oligo(dt) and random hexamer primers) and iScript reverse transcriptase. To check for DNA contamination, controls lacking reverse transcriptase were included. The reverse transcription reaction involved a three-step process: 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. Following the reverse transcription, samples were held at 4°C. The cDNA product synthesized from each sample was assayed for expression levels of each of four tomato genes (acidic *PR-1,* basic *PR-1*, *Pin2*, actin).

Real-time quantitative PCR reactions were carried out in triplicate in 96-well plates. Each reaction (25 µL) was performed using the iQ Supermix Kit (Bio-Rad Laboratories) and consisted of 1X Mastermix, forward and reverse

Table 3.1. Primer and probe sequences used in this study.

Gene	Primer sequence (5'-3') F	Primer sequence (5'-3') R	Probe sequence (5'-3')	References
Actin	TTGCCGCATGCCATTCT	TCGGTGAGGATATTCATCAGGTT	6-FAM/CGTTTGGATCTTGCTGGTCGTGATTTAACT/TAMRA	(This study)
Pin2	TGATGCCAAGGCTTGTACTAGAGA	AGCGGACTTCCTTCTGAACGT	6-FAM/TGTGGTAATCTTGGGTTCGGGATATGCC/TAMRA	(This study)
PR-1a	GAGGGCAGCCGTGCAA	CACATTTTTCCACCAACACATTG	6-FAM/TGTGGGTGTCCGAGAGGCCAGA/BHQ_1	Block et al. [17]
PR-1b	GGTCGGGCACGTTGCA	GATCCAGTTGCCTACAGGACATA	6-FAM/CAACGGATGGTGGTTCATTTCTTGCA/BQH_1	Block et al. [17]
PR-1a[a]	CCCAAAATTCACCCCAAGACT	TCAATCCGATCCCACTTATCATT		(This study)

[a]Primer sequences used in PCR to generate northern probe.

primers (300 nM final concentration) and 200 nM fluorogenic probe. Reaction parameters consisted of 95°C for 4 min, followed by 45 cycles of 95°C for 10 sec, 50°C for 1 min, and 72°C for 30 sec. Controls from the cDNA reaction lacking reverse transcriptase and reactions with no cDNA template were also included.

The standard curve method was used to calculate the initial transcript levels (User Bulletin #2, ABI PRISM 7700 Sequence Detection System, Relative quantification of gene expression, 2001, Applied Biosystems). Standard curves were produced by performing qRT-PCR on serial dilutions of tomato total genomic DNA (Yun et al. 2006). Construction of standard curves was done by plotting the threshold cycle (Ct) against the logarithm of the known concentrations. These curves were used to calculate the absolute quantity of the product in each sample (Ding et al. 2004; Mittapalli et al. 2006). Relative expression values (REVs) were then calculated by normalizing against the tomato actin gene as an internal control (Beaubois et al. 2007). Acibenzolar-*S*-methyl and other salicylic acid-analogs are not known to induce *Pin2* expression, hence REVs normalized to actin are presented (Penacortes et al. 1995).

Analysis of acidic and basic *PR-1* response to ASM application was determined by calibrating the samples to the mean REV of the three replicates (individual field plots) on days 0 and 7 for both the ASM-treated and untreated control plots. Statistical significance was determined using the log of the REVs for each of the three marker genes tested. Values were then analyzed by ANOVA (Analysis of Variance) using the Proc Mixed procedure and SAS software version 9.1(Cary, NC, USA). Orthogonal contrasts were used to

evaluate treatment differences in expression at each time point and *P*-values <0.05 were considered statistically significant as previously described by Mittapalli et al. (Mittapalli et al. 2006). Thus, each bar in Figure 3.1 represents the average REV for the three field plots (a total of 9 qRT-PCR data points) for each cultivar +/- standard error.

Northern blot analysis. Tomato PR genes used as probes for northern analyses included basic *PR-1* and the proteinase inhibitor gene *Pin2*, and were identical to those described by Gu et al. (2000) (Gu et al. 2000). A probe for northern detection of acidic *PR-1* was created by PCR amplifying tomato DNA utilizing primers listed in Table 3.1, followed by amplicon purification using Wizard PCR Preps DNA Purification System (Promega Corporation, Madison, WI). Identity of the fragment was verified via sequencing performed at the Cornell University Bioresource Center. Sample RNA (10 ug) was run on a 1.2% formaldehyde-agarose gel and transferred to Hybond-N membrane (Amersham Biosciences, Piscataway, NJ). The same RNA extractions (described above) were used for both qRT-PCR and northern analyses. Hybridizations were performed as described by Smart et al. (Smart et al. 2003).

RESULTS

ASM induction of jasmonic acid-mediated gene expression. Induction of wound-inducible, jasmonic acid-mediated defenses was measured by the proteinase inhibitor marker gene *Pin2* (Bowles 1998; Fidantsef et al. 1999; Penacortes, Fisahn, and Willmitzer 1995). As expected, *Pin2* expression was not induced by ASM application in any of the cultivars tested (Table 3.2). The *Pin2* gene was induced sporadically in both treated and untreated plants,

Table 3.2. *Pin2* relative expression values for ASM-treated and untreated replicate plots of three tomato cultivars. These values were normalized to tomato actin gene expression.

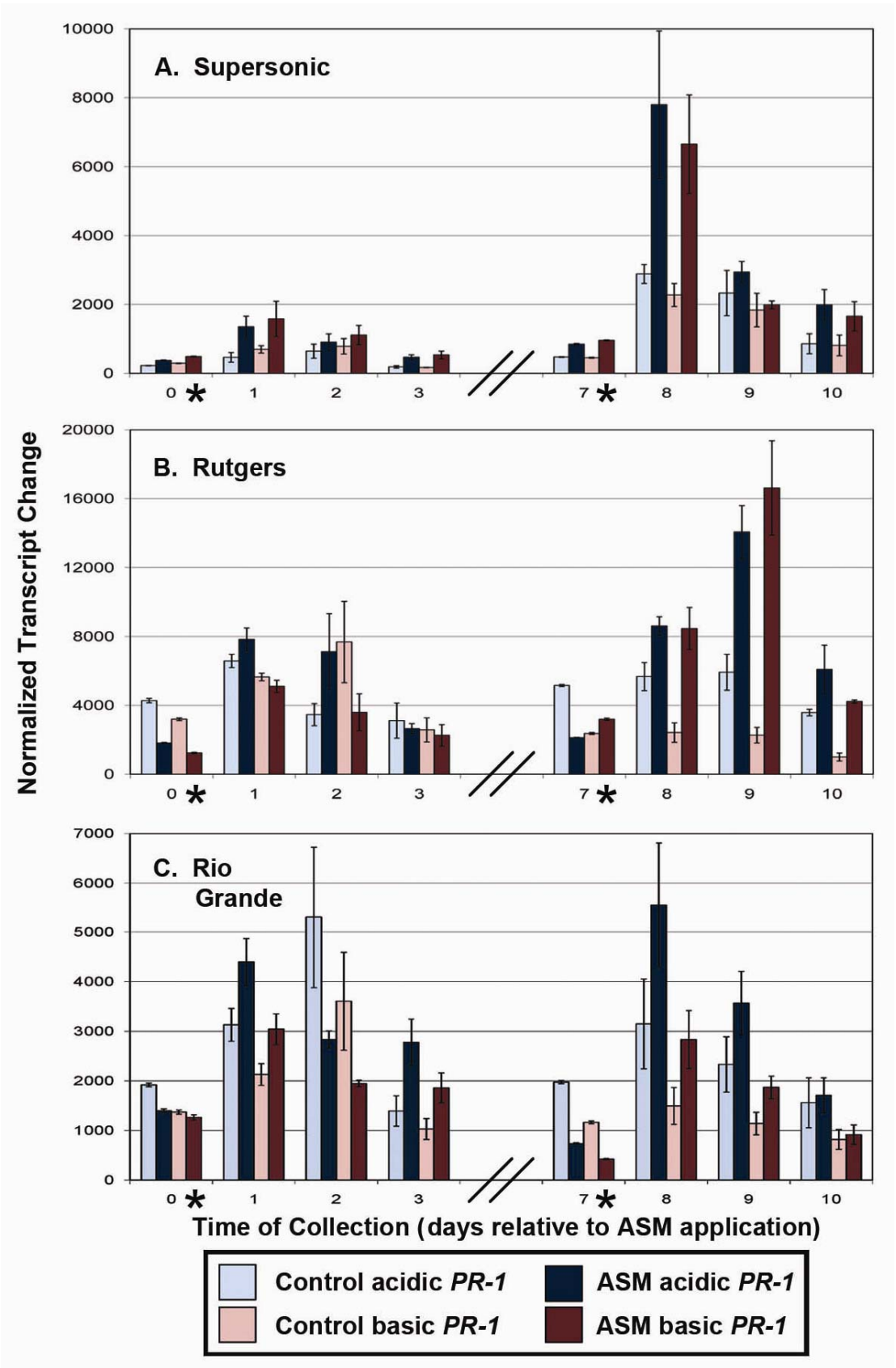
			Days relative to ASM application							
Cultivar	Treatment	Rep	0	1	2	3	7	8	9	10
Supersonic	Control	1	11.75	5.55	10.97	0.56	68.03	16.15	35.55	149.62
		2	8.44	16.79	26.48	6.83	49.05	0.02	5.30	26.84
		3	5.29	0.15	9.79	1.89	37.06	12.05	12.50	0.55
	ASM	1	34.22	35.99	37.68	17.56	191.07	119.88	99.12	129.30
		2	1.77	8.62	2.60	33.64	40.02	0.69	13.18	2.17
		3	2.24	11.66	15.80	6.36	119.37	12.94	0.00	105.24
Rutgers	Control	1	15.97	13.36	6.23	9.50	8827.6	4031.5	2414.9	4870.4
		2	6.13	2536.5	198.00	0.04	115.80	1.74	72.96	31.23
		3	23.19	25.54	17.39	3.38	42.96	7.07	27.61	2.32
	ASM	1	5.33	3.17	3.68	1.86	1518.0	3835.6	5627.3	5021.6
		2	8.37	56.55	44.36	52.28	130.05	164.43	24.18	170.14
		3	2878.7	16.91	18.54	7.35	284.7	5.82	18.47	22.73
Rio Grande	Control	1	15.80	21.85	38.97	98.96	34.42	5.87	26.38	44.40
		2	9.28	822.51	45.18	0.62	28.18	55.40	96.64	217.16
		3	29.67	52.04	19.17	6.84	72.92	126.59	1819.7	62.06
	ASM	1	7.15	59.23	7.89	0.32	40.07	22.92	199.50	31.69
		2	13.81	55.62	65.67	83.53	21.17	2.65	25.76	10.63
		3	3038.7	21.73	28.42	12.06	73.81	14.38	36.68	88.72

usually to a very high level (up to 553 fold increase, Rutgers, control rep 1, day seven). When activated, *Pin2* expression usually dropped to baseline levels the following day (Table 3.2). In a few instances, *Pin2* was induced at low levels for several days (Rio Grande, ASM-treated, rep 2; Rutgers, untreated control, rep 2) or consistently at a high level (Supersonic, ASM-treated, rep 1; Rutgers, control and ASM-treated, rep 1). In these cases application of ASM did not modify expression levels.

ASM induction of salicylic acid-mediated PR gene expression. Acidic *PR-1* is a known marker for salicylic acid-mediated responses and SAR in tomato (Block et al. 2005; Tornero et al. 1997; van Kan et al. 1992). Acidic *PR-1* was induced following application of ASM in all three tomato cultivars tested (Figure 3.1, dark blue bars). This marker gene was induced to a consistently significant level following the second application in all replicates of all cultivars. Fluctuation in acidic *PR-1* expression was observed in untreated control plants in all cultivars (Figure 3.1, light blue bars).

Induction of Supersonic tomatoes. Expression levels of acidic *PR-1* increased one day following the first ASM application in the Supersonic cultivar and decreased to baseline by day 3 (Figure 3.1 A). Acidic *PR-1* expression was 3.6 fold (P=0.0019) higher in ASM-treated plots on day one than on day zero. By day seven of the experiment, acidic *PR-1* expression remained approximately twofold higher than initial levels in both ASM-treated and untreated plants. Similar to the response following the first ASM treatment, the Supersonic cultivar showed greatest acidic *PR-1* expression one day following the second ASM application (day eight). Induction of acidic PR-1 was 9.2 fold ($P<0.0001$) higher in ASM-treated plots, than on day seven

Figure 3.1. Expression patterns of two tomato signaling pathway marker genes in three field-grown tomato cultivars following acibenzolar-S-methyl (ASM) treatment. Top (A), middle (B) and bottom (C) panels represent gene expression in Supersonic, Rutgers and Rio Grande tomato cultivars, respectively. Numbers on the x-axis represent the collection dates following the two ASM applications. Acidic PR-1 expression is represented by light blue bars for untreated plants and dark blue bars denote plants that received ASM treatment. Expression of basic PR-1 is depicted by pink and maroon bars for untreated and ASM-treated plants, respectively. Asterisks (*) denote when ASM treatments were applied, following tissue collection on days zero and seven. Transcript levels were calculated from triplicate data using the standard curve method and normalized to tomato actin expression as an internal control. Bars represent average induction (+/- SE) of gene transcripts in ASM-treated compared to untreated plants averaged over three replicate plots. Gene activation was calibrated to the mean expression value prior to ASM application (days zero and seven) for each treatment group (ASM-treated or untreated control).


A. Supersonic
B. Rutgers
C. Rio Grande
Normalized Transcript Change
Time of Collection (days relative to ASM application)
Control acidic PR-1
Control basic PR-1
ASM acidic PR-1
ASM basic PR-1

(Figure 3.1 A). Thus, activation of salicylic acid-mediated responses followed similar a pattern between the two ASM applications, with a greater level of induction following the second treatment.

Induction of Rutgers tomatoes. Prior to ASM application (day zero), tomatoes in untreated control plots demonstrated twofold higher acidic *PR-1* expression than ASM-treated plots. ASM-treated Rutgers tomatoes showed increased acidic *PR-1* expression (4.3 fold increase, $P<0.0001$) on day one, with expression levels slowly decreasing to near-baseline levels by day three (Figure 3.1 B). Expression of acidic *PR-1* was approximately the same on days zero and seven, just prior to ASM application. Rutgers tomatoes had a different pattern of acidic *PR-1* induction following the second ASM application; peak levels (6.6 fold increase from day seven, $P<0.0001$) occurred on day nine and were characterized by a rapid decrease in expression on day ten (Figure 3.1 B). While Rutgers tomatoes demonstrated greater variability than the other two cultivars in acidic *PR-1* response to ASM, this cultivar also exhibited the highest acidic *PR-1* expression levels.

Induction of Rio Grande tomatoes. Induction of salicylic acid-mediated responses following the first ASM application to Rio Grande tomatoes was observed on day one (3.1 fold increase, $P<0.0001$) and levels decreased slowly (Figure 3.1 C). The untreated control plots of Rio Grande had a sharp increase in both acidic and basic *PR-1* expression on day two. Expression levels of acidic *PR-1* in ASM-treated plots were significantly lower on day seven, prior to the second application, than observed on day zero. Salicylic acid-mediated gene expression was activated on day eight, one day following the second treatment (7.5 fold increase, $P<0.0001$), with expression levels

slowly decreasing after peak activation, similar to the results from the initial spray. Like the other two cultivars, Rio Grande tomatoes showed a greater response to the second ASM application than the first. Of the three cultivars tested, Rio Grande tomatoes demonstrated the most variable acidic *PR-1* expression in untreated control plots.

ASM induction of ethylene-mediated PR gene expression. Ethylene-mediated responses, as measured by expression of the tomato basic *PR-1* gene (Block et al. 2005; Tornero et al. 1997; van Kan et al. 1992), was induced in all cultivars tested following application of ASM, generally with lower expression levels than acidic *PR-1* (Figure 3.1, maroon bars). The general trends of activation were similar between salicylic acid and ethylene-mediated responses in all three cultivars. Prior to ASM application, basic *PR-1* was expressed at low levels in all plants. Basic *PR-1* expression fluctuated in untreated control plants, though expression changes were usually to a much lesser degree than ASM treated plants (Figure 3.1, pink bars). In some instances, levels in untreated plots were elevated consistently over several days (Supersonic cultivar, days seven through ten). In the Rutgers cultivar (days zero through three) and Rio Grande cultivar (on day two only), basic *PR-1* expression in the untreated control was greater than in the ASM-treated plots.

Induction of Supersonic tomatoes. Basic *PR-1* expression increased one day following treatment with ASM in Supersonic tomatoes (3.2 fold increase, P=0.0269) and declined to near-baseline levels over days two and three (Figure 3.1 A). The expression level was approximately twofold higher on day seven then observed on day zero prior to ASM treatment, similar to

that observed for salicylic acid-mediated gene expression. Following the second ASM spray, the highest basic *PR-1* expression levels were observed one day after application (day eight, 7.0 fold increase, $P<0.0001$), and decreased to levels twofold higher than day seven by day ten (Figure 3.1 B). As observed with salicylic acid-mediated responses, activation of ethylene-mediated responses followed a similar pattern after each ASM treatment, with a greater degree following the second ASM application.

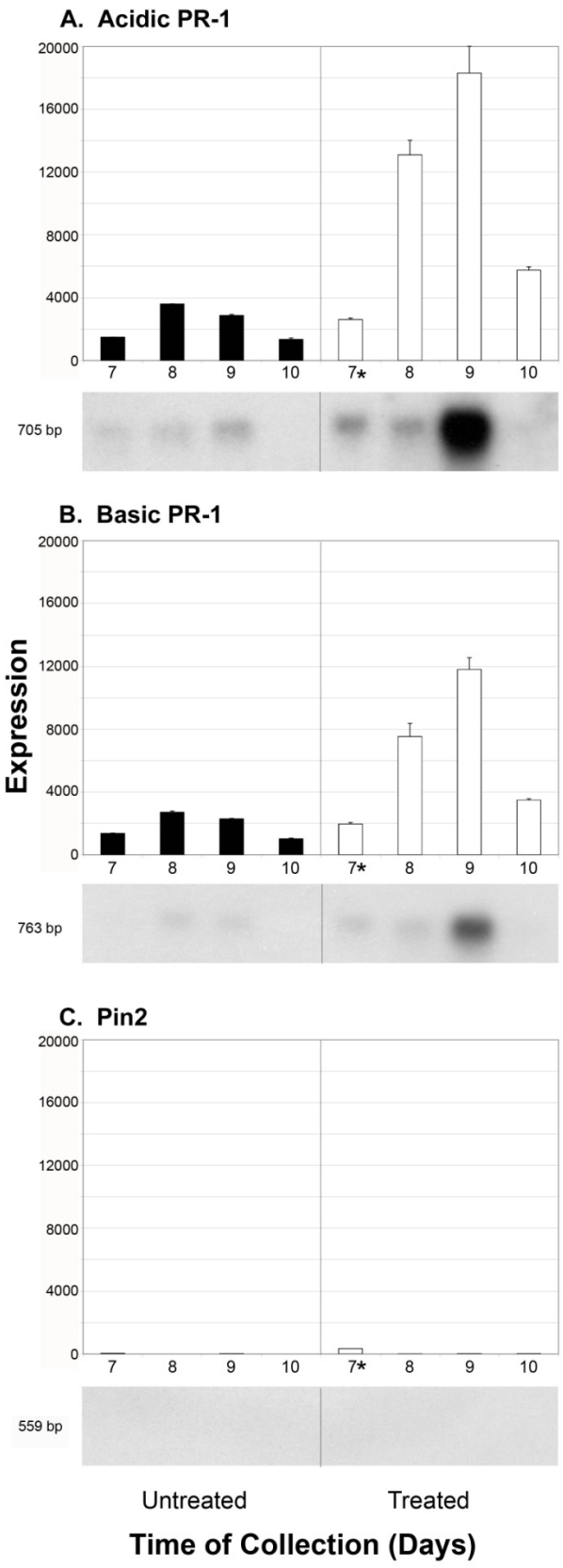
Induction of Rutgers tomatoes. Activation of ethylene-mediated responses in Rutgers tomatoes increased one day following ASM application (4.1 fold increase, $P<0.0001$), though the untreated plants exhibited higher expression levels than the treated plants on days one, two and three (Figure 3.1 B). Expression of basic *PR-1* decreased to levels twofold higher than at time zero by day three in ASM-treated plants. As observed with acidic *PR-1* activation, basic *PR-1* was more highly expressed following the second ASM application. Peak activation occurred on day nine (5.2 fold increase, $P<0.0001$) and declined to levels 1.5 fold greater than day seven on day ten (Figure 3.1 B).

Induction of Rio Grande tomatoes. Induction of ethylene-mediated gene expression in Rio Grande tomatoes occurred one day following ASM application (2.4 fold, $P=0.0011$, Figure 3.1 C), remained elevated until day three, then dropped to below baseline levels prior to the second ASM application (day seven, Figure 3.1 C). Comparable to acidic *PR-1* expression, basic *PR-1* was more highly expressed, relative to untreated control plants, following the second ASM application. Similar expression patterns were observed following the second ASM application, with highest basic *PR-1*

activation one day after treatment (day eight, 6.7 fold increase, $P<0.0001$). Basic *PR-1* levels remained elevated twofold above baseline through day ten (Figure 3.1 C). As seen with cultivar Rutgers, there was significant variation in expression levels in untreated plants during the first three days of the experiment.

Northern validation of qRT-PCR results. Expression patterns observed using northern blot analyses of RNA extracted from tomato field samples (cultivar Rutgers) support findings from the qRT-PCR study. Data shown are from one replicate field plot of Rutgers tomato tissue; similar results were found in all three replicates. A representative northern is shown in Figure 3.2 for tissue collected relative to the second ASM application (days seven through ten). The greatest induction of salicylic acid-mediated gene expression was observed two days following the second ASM application (day nine), with acidic *PR-1* levels decreasing drastically by day ten (Figure 3.2 A). Fluctuations in acidic *PR-1* expression were observed in untreated plants, though levels were significantly lower than those observed in ASM-treated plants. Ethylene-mediated PR gene expression was also activated by ASM application (as measured by basic *PR-1* expression), though at lower levels than the salicylic acid-mediated expression (Figure 3.2 B). Peak expression of basic *PR-1* was observed two days after the second treatment with ASM and markedly decreased by day ten. Untreated plants exhibited low, fluctuating levels of basic *PR-1.* No activation of the jasmonic acid-responsive gene *Pin2* was observed in treated or untreated plants, except at low levels on day seven in ASM-treated plants (Figure 3.2 C). The low level of activation of *Pin2* at this time point was detectable by qRT-PCR, although was below the threshold of

Figure 3.2. Validation of qRT-PCR results using northern blot analysis, comparing ASM-treated and untreated tissue (cultivar Rutgers) from days seven through ten. Transcript levels depicted in the graphs were determined using qRT-PCR and calculated from triplicate data using the standard curve method. Panels A, B, and C refer to the tomato defense response genes acidic *PR-1*, basic *PR-1*, and *Pin2*, respectively. Solid bars indicate results from untreated plants while striped bars indicate results from plants treated with ASM. Asterisks (*) denote when ASM was applied, following tissue collection on day seven. Northern hybridizations, using RNA from the same extraction as that used for qRT-PCR, are shown below the graph. The size (in base pairs) of each defense gene mRNA is indicated to the left of each panel. Bottom panel (rRNA) shows ethidium bromide stained gel with equal loading of each sample.


A. Acidic PR-1
20000
16000
12000
8000
4000
0
7
8
9
10
7*
8
9
10
705 bp
B. Basic PR-1
Expression
763 bp
C. Pin2
559 bp
Untreated
Treated
Time of Collection (Days)

detection by northern blotting. The qRT-PCR results shown in Figure 3.2 are not identical to those shown in Figure 3.1, as they represent a single field plot replicate whereas Figure 3.1 presents the combined data from all three replicates.

DISCUSSION

While plant activators have been extensively studied in the greenhouse and growth chamber (Kohler et al. 2002; Potlakayala et al. 2007; Ryu et al. 2003), this is the first experiment following activator induction in multiple tomato cultivars over time in the field. Importantly, we demonstrated that a commercially available SAR-inducing compound effectively activated tomato defense responses, though there was some variation in cultivar response.

Field experiments performed by Thaler et al. found BTH consistently induced SAR in field tomatoes five days after application, though not to levels significantly higher than the untreated control (Thaler et al. 1999). Other research has examined plant activator effects in tobacco grown in the greenhouse, determining that acidic *PR-1* was activated twelve hours and basic *PR-1* three days after application, with both genes maintaining high levels of expression up to twenty days after treatment (Friedrich et al. 1996). Potlakayala et al. found that BTH induced *PR-1* in greenhouse-grown canola starting one day after application, and remained upregulated for up to three weeks prior to pathogen challenge (Potlakayala et al. 2007). It is intriguing that ASM-induced defense gene responses in the three tomato cultivars tested in this experiment decreased to baseline levels by seven days following application (Figs. 3.1 and 3.3), a different result from the response of greenhouse-grown canola and tobacco. Levels of ASM were measured in

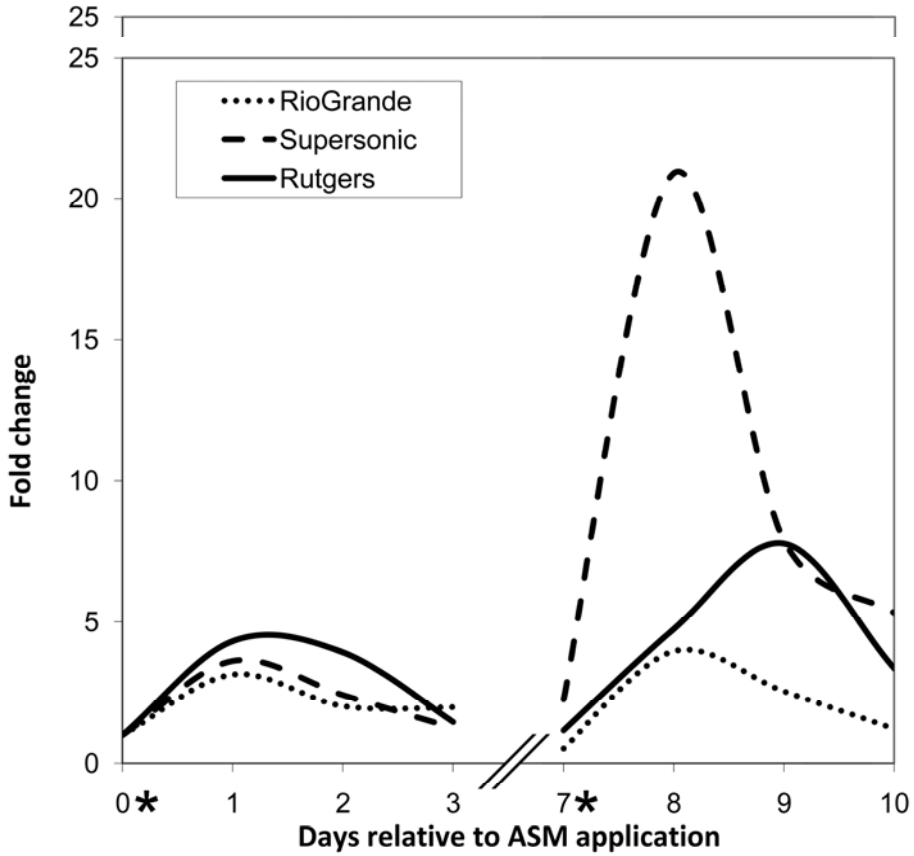


Figure 3.3. Compilation of data depicting the fold change of acidic *PR-1* expression (which serves as a marker gene for the salicylic acid signaling pathway) for each tomato cultivar throughout the time course of the experiment. Changes are shown as the fold-change of acidic *PR-1* compared to untreated plants on day zero. Asterisks (*) denote when ASM treatments were applied (immediately following tissue collection on days zero and seven).

growth chamber-grown tomatoes using HPLC analysis and were found to peak and return to baseline between two and seventy-two hours after treatment (Scarponi, Buonaurio, and Martinetti 2001). It is interesting that despite the variability in activation of salicylic acid-mediated responses among the three cultivars in this study, acidic *PR-1* expression patterns also peaked and returned to baseline within this time frame.

Environmental conditions in the field were normal over the course of the experiment, with an average temperature of 26.7°C during the day and 16.7°C at night. Over the eleven days of the experiment, 10.0 cm of rain fell though there were no storm or hail events. Wound induced jasmonic acid-mediated responses were not turned on in all plants by any weather event during the experiment. In fact, no pattern was observed in wound-induced (*Pin2*) expression in any tomato cultivar (Table 3.2). Periodically this gene was activated, presumably due to insect, mammal or environmental damage.

While three tomato signaling pathways were monitored in this study, we were most interested in activation of salicylic acid-mediated responses, which are characterized by an increase in acidic *PR-1* expression, as ASM induces SAR (Block et al. 2005; Tornero et al. 1997; van Kan et al. 1992). Figure 3.3 depicts the fold-change induction of the three cultivars tested (relative to day zero) over the course of two ASM applications in the field. Interestingly, among the three cultivars tested, induction of salicylic acid-mediated PR gene expression exhibited a similar pattern following the first ASM application, with all three cultivars exhibiting 2.5-5 fold increases from day zero, one day after treatment (Figure 3.3). Although acidic *PR-1* was clearly induced in Supersonic tomatoes, the effect of ASM on the Rutgers and Rio Grande

cultivars was confounded by significant acidic *PR-1* expression in untreated control plants (Figure 3.1). All cultivars exhibited near-baseline expression levels by seven days after ASM application. Following the second ASM application, all cultivars exhibited a much more significant response relative to untreated control plants. Acidic *PR-1* was consistently induced in the Supersonic cultivar one day following treatment, with drastically higher activation following the second ASM application (Figure 3.3). Rio Grande tomatoes responded in a similar fashion independent of spray timing, though there was much less fluctuation in the untreated control plants after the second ASM treatment. In contrast to Supersonic and Rio Grande cultivars, where activation of SAR followed a similar pattern between the two ASM applications, Rutgers tomatoes responded more slowly, but were induced to a greater level, following the second ASM treatment. Activation was delayed one day in the Rutgers cultivar, as peak expression was on day nine.

Real-time monitoring of gene expression in the field was validated with northern blot analysis (Figure 3.2). Similar findings between the two methods indicate that the qRT-PCR results are accurate and no cross hybridization occurred. These data also demonstrate that qRT-PCR could detect small fluctuations in gene activation that were below the detection threshold of northern blot analysis (Figure 3.2C). This is consistent with previous finding that qRT-PCR is significantly more sensitive than northern analysis (Bustin 2000).

Results of this study provide fundamental information on the defense signaling involved with use of the plant activator ASM under field conditions. Variability in SAR activation among the three cultivars tested offers insight into

the complexities of plant activator-tomato interactions. Tomato cultivar differences have been shown to affect the level of insect damage and degree of insect control from insecticides (Schuster 1977). In sunflower, ASM induced resistance to the parasitic plant, *Orobanche cumana* (broomrape), but level of control was cultivar dependant (Fan et al. 2007). Knowing that field-grown tomato cultivars differ in both base-line levels of acidic and basic *PR-1* levels as well as in the level of activation will enable future studies to determine if ASM application will have a larger impact on disease control in cultivars such as Supersonic, with a dramatic fold-increase in acidic *PR-1* expression following the second treatment. Additionally, studying signaling pathway activation by monitoring marker gene expression following the application of a plant activator and inoculation with a pathogen is necessary to determine if pathogen attacks trigger the re-activation of plant defenses. These pathogen-plant activator interactions will further enhance our ability to integrate plant activators into disease control strategies.

REFERENCES

Anith, K. N., Momol, M. T., Kloepper, J. W., Marois, J. J., Olson, S. M., and Jones, J. B. 2004. Efficacy of plant growth-promoting rhizobacteria, acibenzolar-*S*-methyl, and soil amendment for integrated management of bacterial wilt on tomato. *Plant Disease* 88 (6):669-673.

Baysal, O., Soylu, E. M., and Soylu, S. 2003. Induction of defense-related enzymes and resistance by the plant activator acibenzolar-*S*-methyl in tomato seedlings against bacterial canker caused by *Clavibacter michiganensis* ssp *michiganensis*. *Plant Pathology* 52 (6):747-753.

Beaubois, E., Girard, S., Lallechere, S., Davies, E., Paladian, F., Bonnet, P., Ledoigt, G., and Vian, A. 2007. Intercellular communication in plants: Evidence for two rapidly transmitted systemic signals generated in response to electromagnetic field stimulation in tomato. *Plant Cell and Environment* 30 (7):834-844.

Block, A., Schmelz, E., O'Donnell P, J., Jones, J. B., and Klee, H. J. 2005. Systemic acquired tolerance to virulent bacterial pathogens in tomato. *Plant Physiology* 138 (3):1481-1490.

Bowles, D. 1998. Signal transduction in the wound response of tomato plants. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 353 (1374):1495-1510.

Bustin, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 25 (2):169-93.

Ding, J. Y., Jia, J. W., Yang, L. T., Wen, H. B., Zhang, C. M., Liu, W. X., and Zhang, D. B. 2004. Validation of a rice specific gene, sucrose phosphate synthase, used as the endogenous reference gene for qualitative and real-time quantitative PCR detection of transgenes. *Journal of Agricultural and Food Chemistry* 52 (11):3372-3377.

Durrant, W. E., and Dong, X. 2004. Systemic acquired resistance. *Annual Review of Phytopathology* 42:185-209.

Fan, Z. W., Buschmann, H., Muller-Stover, D., and Sauerborn, J. 2007. Main effects and interactions among acibenzolar-*S*-methyl, a biocontrol fungus and sunflower cultivar on control of *Orobanche cumana* Wallr. *Journal of Plant Diseases and Protection* 114 (2):76-81.

Fidantsef, A. L., Stout, M. J., Thaler, J. S., Duffey, S. S., and Bostock, R. M. 1999. Signal interactions in pathogen and insect attack: expression of lipoxygenase, proteinase inhibitor II, and pathogenesis-related protein

P4 in the tomato, *Lycopersicon esculentum*. *Physiological and Molecular Plant Pathology* 54 (3-4):97-114.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M. G., Meier, B., Dincher, S., Staub, T., Uknes, S., Metraux, J. P., Kessmann, H., and Ryals, J. 1996. A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant Journal* 10 (1):61-70.

Graves, A.S., and Alexander, S.A. 2002. Managing bacterial speck and spot of tomato with acibenzolar-*S*-methyl in Virginia. online. *Plant Health Progress*. doi:10.1094/PHP-2002-0220-01-RS.

Gu, Y. Q., Yang, C., Thara, V. K., Zhou, J., and Martin, G. B. 2000. Pti4 is induced by ethylene and salicylic acid, and its product is phosphorylated by the Pto kinase. *Plant Cell* 12 (5):771-86.

Kohler, A., Schwindling, S., and Conrath, U. 2002. Benzothiadiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the NPR1/NIM1 gene in Arabidopsis. *Plant Physiology* 128 (3):1046-1056.

Kuc, J. 2001. Concepts and direction of induced systemic resistance in plants and its application. *European Journal of Plant Pathology* 107:7-12.

Louws, F. J., Wilson, M., Campbell, H. L., Cuppels, D. A., Jones, J. B., Shoemaker, P. B., Sahin, F., and Miller, S. A. 2001. Field control of bacterial spot and bacterial speck of tomato using a plant activator. *Plant Disease* 85 (5):481-488.

Mittapalli, O., Shukle, R.H., Sardesai, N., Giovanini, M.P., and Williams, C.E. 2006. Expression patterns of antibacterial genes in the Hessian fly. *Journal of Insect Physiology* 52:1143-1152.

Oostendorp, M., Kunz, W., Dietrich, B., and Staub, T. 2001. Induced disease resistance in plants by chemicals. *European Journal of Plant Pathology* 107 (1):19-28.

Penacortes, H., Fisahn, J., and Willmitzer, L. 1995. Signals involved in wound-induced proteinase-inhibitor-II gene-expression in tomato and potato plants. *Proceedings of the National Academy of Sciences of the United States of America* 92 (10):4106-4113.

Perry, K. L., and Francki, R. I. 1992. Insect-mediated transmission of mixed and reassorted cucumovirus genomic RNAs. *J Gen Virol* 73:2105-14.

Potlakayala, S.D., Reed, D.W., Covello, P.S., and Fobert, P.R. 2007. Systemic acquired resistance in canola is linked with pathogenesis-related gene expression and requires salicylic acid. *Phytopathology* 97 (7):794-802.

Pradhanang, P. M., Ji, P., Momol, M. T., Olson, S. M., Mayfield, J. L., and Jones, J. B. 2005. Application of acibenzolar-*S*-methyl enhances host resistance in tomato against *Ralstonia solanacearum*. *Plant Disease* 89 (9):989-993.

Reiners, S., and Petzoldt, C., eds. 2007. *The integrated crop and pest management guidelines for vegetables*. Ithaca: Cornell University.

Ryu, C. M., Hu, C. H., Reddy, M. S., and Kloepper, J. W. 2003. Different signaling pathways of induced resistance by rhizobacteria in *Arabidopsis thaliana* against two pathovars of *Pseudomonas syringae*. *New Phytologist* 160 (2):413-420.

Scarponi, L., Buonaurio, R., and Martinetti, L. 2001. Persistence and translocation of a benzothiadiazole derivative in tomato plants in relation to systemic acquired resistance against *Pseudomonas syringae* pv *tomato*. *Pest Manag Sci* 57 (3):262-8.

Schuster, D. J. 1977. Effect of tomato cultivars on insect damage and chemical control. *Florida Entomologist* 60 (3):227-232.

Smart, C. D., Myers, K. L., Restrepo, S., Martin, G. B., and Fry, W. E. 2003. Partial resistance of tomato to *Phytophthora infestans* is not dependent upon ethylene, jasmonic acid, or salicylic acid signaling pathways. *Molecular Plant-Microbe Interactions* 16 (2):141-148.

Thaler, J. S., Fidantsef, A. L., Duffey, S. S., and Bostock, R. M. 1999. Trade-offs in plant defense against pathogens and herbivores: A field demonstration of chemical elicitors of induced resistance. *Journal of Chemical Ecology* 25 (7):1597-1609.

Tornero, P., Gadea, J., Conejero, V., and Vera, P. 1997. Two *PR-1* genes from tomato are differentially regulated and reveal a novel mode of expression for a pathogenesis-related gene during the hypersensitive response and development. *Mol Plant Microbe Interact* 10 (5):624-34.

van Kan, J. A., Joosten, M. H., Wagemakers, C. A., van den Berg-Velthuis, G. C., and de Wit, P. J. 1992. Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum*. *Plant Molecular Biology* 20 (3):513-27.

Walters, D. R., and Boyle, C. 2005. Induced resistance and allocation costs: What is the impact of pathogen challenge? *Physiological and Molecular Plant Pathology* 66 (1-2):40-44.

Wilson, M., Campbell, H. L., Ji, P., Jones, J. B., and Cuppels, D. A. 2002. Biological control of bacterial speck of tomato under field conditions at several locations in North America. *Phytopathology* 92 (12):1284-1292.

Yun, J. J., Heisler, L. E., Hwang, I. I. L., Wilkins, O., Lau, S. K., Hyrcza, M., Jayabalasingham, B., Jin, J., McLaurin, J., Tsao, M. S., and Der, S. D. 2006. Genomic DNA functions as a universal external standard in quantitative real-time PCR. *Nucleic Acids Research* 34 (12):E85-E85.

CHAPTER FOUR

IMPACT OF PLANT ACTIVATORS AND COPPER ON BACTERIAL SPECK AND GENE EXPRESSION IN FIELD-GROWN TOMATOES

ABSTRACT

Two types of plant activators and copper were evaluated for control of bacterial speck disease and tomato defense gene activation over three field seasons. Acibenzolar-*S*-methyl (ASM), which activates systemic acquired resistance, controlled *Pseudomonas syringae* pv. *tomato* as well as copper and no negative effects on yield were observed. The plant growth-promoting rhizobacteria (PGPR, activator of induced systemic resistance) compound reduced bacterial speck symptoms relative to the untreated control though did not consistently control to the level of ASM and copper. Alone or in combination with ASM, the PGPR compound provided some boost in yield in one of the three years of the trial. All treatments negatively impacted pathogen growth. Response of ASM-treated plants was dependant on disease pressure; the salicylic acid and ethylene signaling pathways were activated to detectable levels only under high disease pressure. Despite providing some disease control, no priming of signaling pathways was observed in PGPR-treated plants. Implications of these findings on field management strategies and defense pathway interactions are discussed.

INTRODUCTION

Bacterial speck disease of tomato, caused by *Pseudomonas syringae* pv. *tomato*, is a economically important bacterial disease found world-wide in tomato-growing regions (Goode and Sasser 1980). Yield losses occur under optimal environmental conditions for pathogen growth, cool temperatures (18-24°C) and high relative humidity (Goode and Sasser 1980; Jones et al. 1991; Louws et al. 2001). Symptomatic plants typically exhibit small, necrotic lesions surrounded by chlorotic halos on foliage and fruit, though all aboveground plant parts are susceptible. Though this disease does not often kill the plant, yield losses can occur from decreased marketability of symptomatic fruit (Yunis et al. 1980).

Currently, bacterial speck is controlled by cultural methods and copper-based bactericides (Reiners and Petzoldt 2007; Smart et al. 2005). Deployment of tomato cultivars containing the *Pto* resistance gene has also been an effective control measure for bacterial speck in processing tomatoes (Pedley and Martin 2003). Despite the relatively successful use of current control measures, there is a need for additional disease management tools. Copper resistant strains of *P. syringae* pv. *tomato* have been reported, though not in New York (Alexander et al. 1999; Bender and Cooksey 1986; Cuppels and Elmhirst 1999; Scheck et al. 1996; Silva and Lopes 1995) and environmental concerns are raised by excessive use of heavy metals in agriculture (He et al. 2005). Races of *P. syringae* pv. *tomato* that can overcome *Pto*-mediated resistance have also been reported (Buonaurio et al. 1996; Lawton and Macneill 1986).

Plant activators, compounds that can activate plant defense responses and control disease without directly impacting the pathogen, could be a valuable tool in a management program by delaying initial pesticide applications or they could be alternated with chemical control. Additionally, many of these products claim to increase plant health and yield and have a lower environmental impact. Resistance induced by plant activators involves stimulation of innate plant defenses, which in turn enables plants to respond against later pathogen attack (Vallad and Goodman 2004; van Loon et al. 1998). There are two well characterized induced resistance pathways that elicit phenotypically similar responses, with distinct regulatory pathways. One type of induced resistance is systemic acquired resistance (SAR), which involves salicylic acid signaling (Durrant and Dong 2004; Sticher et al. 1997). Induced systemic resistance (ISR) involves living microbes that activate a resistance mechanism independently of salicylic acid and constitute the second type of induced resistance pathway (Bostock 2005; Kloepper, Ryu et al. 2004; Persello-Cartieaux et al. 2003).

Systemic acquired resistance-inducing compounds such as acibenzolar-*S*-methyl (ASM) have been found to control bacterial speck as well as copper, though there have been some reports of yield reduction (Louws et al. 2001; Stout et al. 1998). Plant growth-promoting rhizobacteria (PGPR), specifically *Bacillus* spp., have been shown activate induced systemic resistance (ISR) while increasing plant health and yield (Kloepper, Ryu, and Zhang 2004; Kloepper, Reddy et al. 2004). Additionally, some PGPR compounds effectively control tomato diseases alone, or in combination with SAR-inducing compounds (Anith et al. 2004; Domenech et al. 2006; Guo

et al. 2004; Jetiyanon et al. 2003; Murphy et al. 2003). Further field studies, under varying environmental conditions, are necessary to determine if there is a physiological toll due to SAR that is reflected by poor yield and/or crop quality, or if PGPR consistently increase yield (Dietrich et al. 2005). Activators need to be assessed in agricultural growing systems to fully understand their impact on yield.

The objective of this study was to synthesize information on the impact of plant activators and copper on *P. syringae* pv. *tomato,* yield and plant defense gene activation to evaluate whether plant activators could be effectively incorporated into a disease management strategy in New York. Additionally, it is unknown whether crosstalk between defense response pathways occurs when two plant activators are used together and whether they could act synergistically to enhance both yield and disease control. Studying defense pathway activation following the application of control compounds and pathogen inoculation is necessary to determine whether pathogen attacks trigger the re-activation of plant defenses under field conditions. Knowledge of these pathogen-plant activator interactions will further enhance our ability to integrate plant activators into disease control strategies in New York.

MATERIALS AND METHODS

Plant materials and treatments. Tomato seeds (*Solanum lycopersicon* cv. Sunchief VF) were sown in 128 cell polystyrene flats with a 4:1:1 blend of Cornell mix (a soiless peat mixture), perlite and vermiculite. The PGPR treatment, BioYield Concentrate (Bayer CropScience, Research Triangle Park, NC), was mixed evenly into the soil at this time (1.2 kg/m3). *Bacillus subtilis*

GB03 and *B. amyloliquefaciens* IN937a are the two bacterial strains present in this PGPR compound at a concentration of 5.0e9 endospores per cm^3. Plants were grown in the greenhouse under natural sunlight (15/9 h L/D) with temperatures of 23-26°C (day) and 20-22°C (night). Seedlings were moved to an outdoor coldframe one week prior to field planting.

Field trials were conducted over the summer in Geneva, NY in 2004-2006. Each year, the field was rotated, cultivated and 136 kg of 15-15-15 (N-P-K) fertilizer was incorporated at a rate of 50.4 kg N per ha prior to planting. Six-week old seedlings were mechanically transplanted into raised beds covered with 1.25 mm black polyethylene. Starter fertilizer (21-5-20) at a rate of 1 kg / 208 L water was incorporated at planting. Five treatments (untreated control, PGPR, ASM, ASM+PGPR, and copper) were arranged in a randomized complete block design with three replicates. Each plot consisted of twenty plants, divided evenly between two rows spaced 2 m apart. Individual plants were spaced 45.7 cm apart with 1.5 m between plots. Typical production practices for western NY were followed for tomato fertilization, irrigation and weed control (Reiners and Petzoldt 2007). In 2004, all plots were inoculated, whereas in 2005 and 2006, one row within each plot received the pathogen and the other remained uninoculated.

Acibenzolar-*S*-methyl (Actigard, Syngenta Crop Protection, Greensboro, NC) and copper (Cuprofix 40 disperss, Cerexagri, Inc., King of Prussia, PA) treatments were applied with a CO_2 pressurized backpack sprayer on a seven day spray schedule. Both Actigard and Cuprofix were applied according to labeled rates, 52 g a.i./ha and 2.8 kg a.i./ha, respectively.

Foliar treatments began two weeks prior to pathogen inoculation and continued until fruit were harvested.

Pathogen inoculation and disease rating. Pseudomonas syringae pv. *tomato* strain A9 was grown overnight and shaken at 180 rpm at 28ºC in nutrient broth (Schaad et al. 2001). Five ml of this inoculum was then transferred to flasks containing 500 ml of nutrient broth and again grown overnight with shaking. Bacteria were spun down at 20,000 x *g* for 15 minutes and rinsed with 10 mM $MgCl_2$ prior to inoculation. Subsequently, bacteria were resuspended (10^8 CFU/ml) in 10 mM $MgCl_2$ containing 200µl/L of the surfactant Silwet L77 (Helena Chemical, Collierville, TN) and applied to runoff using a pump sprayer. Prior to inoculation, foliage was misted with water and uninoculated plants were sprayed with 10 mM $MgCl^2$ containing 200 µl/L Silwet L77. Pathogen inoculation was carried out at 7:00 PM, whereas all tissue collection for gene expression analysis (see below) was performed at 7:00 AM.

Plants were inoculated four times (29 Jul and 4, 12, 18 Aug) in 2004 and twice in 2005 (9 and 23 Aug). In 2006, plants were inoculated on 20 Jul following a damaging hail storm with high winds.

Foliar symptoms of bacterial speck were evaluated via removing 20 random leaflets per plot and counting the number of lesions. Symptom assessment was performed 28 and 17 days following the first inoculation in 2004 and 2005, respectively. In 2006, disease was rated twice, both 4 and 8 days following inoculation. Mean number of bacterial speck lesions per leaflet was evaluated using one-way ANOVA (SAS software version 9.1, Cary, NC, USA) and significant differences between means were separated using Fisher's LSD at P=0.05. Bacterial speck symptoms were first analyzed

separately by year, then with data from the three years combined. In 2006, fruit symptoms were severe and the number of fruit exhibiting lesions was recorded. Proportion of symptomatic fruit was assessed using a one-way ANOVA (SAS software 9.1) and means compared with a Fisher's LSD at P=0.05.

Bacterial speck quantification. Bacterial populations on leaf tissue samples from the 2006 field season were enumerated using quantitative real-time PCR (qRT-PCR). Total DNA was extracted using the Qiagen DNeasy Kit (Qiagen, Inc., Madison, WI). Real-time quantitative PCR reactions containing 160 ng DNA of each sample were carried out in triplicate in 96-well plates. Each reaction (25 µL) used the iQ SYBR Green Supermix (Bio-Rad Laboratories) and contained 1X Mastermix plus *P. syringae* pv. *tomato*-specific primers from the *hrpZ* gene, MM5F and MM5R (300 nM final concentration). Primers MM5F and MM5R as well as PCR conditions were identical to those described by Zaccardelli et al. (Zaccardelli et al. 2005). A standard curve generated from 10-fold dilutions of *P. syringae* pv. *tomato* DNA was used to quantify bacterial DNA in plant tissue samples (Brouwer et al. 2003).

Yield evaluation. Each field season, tomato fruit from each treatment larger than 5 cm were harvested from five plants per plot, counted, sorted by size class and weighed. Late blight, caused by *Phytophthora infestans,* was discovered in the field in late Aug 2004. This pathogen is a significant threat to local growers, so the trial was ended prior to fruit maturity and green fruit harvested on 2 Sep. Marketable fruit were harvested on 29 Aug and 13 Sept 2005. Due to severe hail damage in 2006, none of the fruit on any plants were marketable. Additionally, wounds in the fruit increased the opportunity for

secondary pathogen infection. Thus, total fruit was harvested on 22 Aug. All yield data were analyzed using a one-way ANOVA (SAS software 9.1) and differences between means were separated using Fisher's LSD at *P*=0.05. Yield was analyzed separately by year, then in combination.

Tissue collection and RNA extraction. Two leaves (each with 5-7 leaflets) were collected from individual field plots for each of three replicates and flash frozen in liquid nitrogen. Tomato tissue was collected at five time points relative to inoculation with *P. syringae* pv. *tomato*, immediately prior to inoculation (-12 h), 12, 36, and 60 hours following and at symptom development (Figure 4.1). Leaf tissue was stored at -80°C until processed for RNA extraction. Total tomato RNA was isolated from leaf tissue using the SV Total RNA Isolation System (Promega Corporation, Madison, WI) and further DNase treated with Turbo DNA-*free* (Ambion Inc., Austin, TX). RNA was checked for degredation by separating 1µg electrophoretically on a 1.2% agarose formaldehyde gel.

Quantification of gene expression using quantitative real-time PCR (qRT-PCR). Primers and fluorogenic probes used were identical to those used in Herman et al. 2008 (see chapter 3). Marker genes for the salicylic acid (acidic *PR-1*), ethylene (basic *PR-1*), and jasmonic acid (*Pin2*) signaling pathways were followed throughout the time course of the experiment and expression values were normalized to the housekeeping gene actin. The iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories) was used to carry out two-step qRT-PCR. Complementary DNA (cDNA) was generated from total RNA (2 µg) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories)

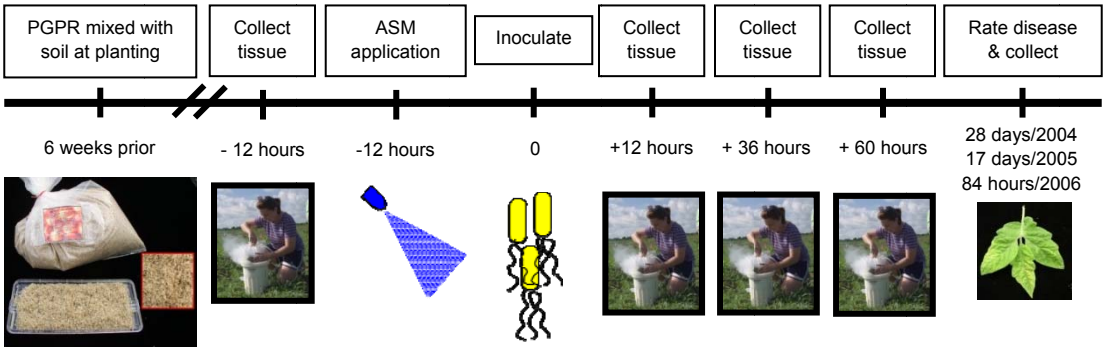


Figure 4.1. Time line of treatment application, pathogen inoculation and tissue collection. Timing is relative to inoculation with *Pseudomonas syringae* pv. *tomato*.

for the first step. Expression levels of each of the four tomato genes were then determined from the cDNA product synthesized from each sample. Real-time quantitative PCR reactions were carried out in triplicate in 96-well plates. Each reaction (25 µL) was performed using the iQ Supermix Kit (Bio-Rad Laboratories) and consisted of 1X Mastermix, forward and reverse primers (300 nM final concentration) and 200 nM fluorogenic probe. Reaction parameters consisted of 95°C for 4 min, followed by 45 cycles of 95°C for 10 sec, 50°C for 1 min, and 72°C for 30 sec.

The standard curve method was used to calculate the initial transcript levels (User Bulletin #2, ABI PRISM 7700 Sequence Detection System, Relative quantification of gene expression, 2001, Applied Biosystems). Standard curves were produced by performing qRT-PCR on serial dilutions of tomato total genomic DNA (Yun et al. 2006). Construction of standard curves was done by plotting the threshold cycle (Ct) against the logarithm of the known concentrations. These curves were used to calculate the absolute quantity of the product in each sample (Ding et al. 2004; Mittapalli et al. 2006). Relative expression values (REVs) were then calculated by normalizing against the tomato actin gene as an internal control (Beaubois et al. 2007).

Analysis of defense gene responses to pathogen inoculation was performed by calibrating the samples to the mean REV of the three replicates (individual field plots) at -12 h for both the inoculated and uninoculated plots. The log REVs for each of the three marker genes tested was analyzed using a one-way ANOVA (SAS software 9.1). Orthogonal contrasts were used to evaluate treatment differences in expression at each time point and *P*-values <0.05 were considered statistically significant (Mittapalli et al. 2006). Thus,

each bar in Figs. 2-7 represents the average REV for the three field plots (a total of 9 qRT-PCR data points) for each treatment +/- standard error.

RESULTS

Effect of treatments on bacterial speck disease symptoms. Bacterial speck severity, as measured by the number of lesions per leaflet, was analyzed separately for each of three years, then trends assessed with the data combined. In 2004, ASM, alone or in combination with the PGPR compound, and copper-treated plants had significantly fewer foliar lesions than the untreated control (P<0.003, Table 4.1). The PGPR compound alone provided no significant control of bacterial speck (P=0.3228).

Although disease pressure in 2005 was minimal in the field, all treatments significantly reduced the number of bacterial speck lesions compared to the untreated control (P<0.0001, Table 4.2). Plants treated with both ASM and the PGPR compound had the lowest disease severity, though not significantly different from copper or ASM alone (P=0.3691, P=0.2443, respectively). No symptoms were ever observed in any of the noninoculated plots.

Plants exhibited extreme wind and hail damage in 2006. Bacterial speck symptoms were severe across all treatments, although the number of lesions in all treated plots was significantly lower than the untreated control four and eight days after inoculation (P<0.03, P <0.0001, respectively, Table 4.3). Both ASM treatments and copper provided significantly better control of *P. syringae* pv. *tomato* than the PGPR compound alone. Though all treatments reduced foliar symptoms of bacterial speck, none significantly

Table 4.1. Effect of plant activators and copper on bacterial speck disease and tomato yield, 2004.

Treatment	Lesion no.[ab]	Fruit no.[c]	Fruit wt[d]
Control	34.0 a	103.3 b	15.5 b
PGPR	41.3 a	145.3 a	22.1 a
ASM	2.7 b	103.0 b	16.3 b
ASM+PGPR	0.3 b	105.7 b	18.0 b
Copper	4.3 b	111.3 b	17.8 b

[a]Mean number of lesions on 20 leaflets per plot (averaged over three replicates).

[b]Means within a column sharing the same letters are not significantly different (P>0.05, Fisher's LSD).

[c]Mean number of fruit on 5 plants per plot.

[d]Mean weight (kg) of fruit from 5 plants per plot.

Table 4.2. Effect of plant activators and copper on bacterial speck disease and tomato yield, 2005

Treatment	Lesion no.[ab]	Fruit no.[c]	Fruit wt[d]	Fruit no.	Fruit wt
		29 Aug		13 Sept	
Control	15.9 a	14.9 a	4.7 a	15.6 a	3.6 a
PGPR	5.9 b	14.9 a	4.5 a	13.8 a	3.3 a
ASM	4.3 bc	11.7 a	4.1 a	14.5 a	3.7 a
ASM+PGPR	2.1 c	11.2 a	4.3 a	14.0 a	3.4 a
Copper	3.8 bc	13.1 a	4.3 a	16.7 a	4.0 a

[a]Mean number of lesions on 20 leaflets per plot (averaged over three replicates).

[b]Means within a column sharing the same letters are not significantly different (P>0.05, Fisher's LSD)

[c]Mean number of fruit on 5 plants per plot.

[d]Mean weight (kg) of fruit from 5 plants per plot.

Table 4.3. Effect of plant activators and copper on bacterial speck disease, 2006.

Treatment	Lesion no.[ab] 4 days	Lesion no. 8 days	π fruit infected[c]
Control	13.3 a	79.6 a	0.62 a
PGPR	10.5 b	59.1 b	0.76 a
ASM	5.8 c	24.8 c	0.59 a
ASM+PGPR	6.7 c	25.7 c	0.56 a
Copper	7.9 c	32.5 c	0.59 a

[a]Mean number of lesions on 20 leaflets per plot (averaged over three replicates).

[b]Means within a column sharing the same letters are not significantly different (P>0.05, Fisher's LSD).

[c]Mean proportion of fruit exhibiting bacterial speck symptoms.

reduced the proportion of symptomatic fruit (P=0.6344, Table 4.3). No symptoms were ever observed in any of the noninoculated plots.

Bacterial speck severity was significantly lower in all treatments relative to the untreated control when data were combined and analyzed over the three field seasons (P<0.0001, Table 4.4). The PGPR compound alone significantly reduced bacterial speck symptoms, but did not provide the same level of control as ASM or copper-treated plants. While ASM and ASM plus PGPR-treated plants had the lowest severity, they were not significantly different from the copper treatment (P=0.1831, P=0.0970).

Effect of treatments on bacterial speck populations. No bacterial DNA was detected in any of the plots prior to inoculation (Figure 4.2). Untreated control plants exhibited increasing amounts of the pathogen, with highest amounts detected at symptom development (84 hours, 2 fold higher than any other treatments). The greatest amount of pathogen DNA was detected at symptom development (84 hours post inoculation) in PGPR, ASM and copper-treated plants (2, 3.5 and 2.8 fold lower than untreated control plants). The quantity of *P. syringae* pv. *tomato* DNA increased up to 36 hours in ASM and PGPR-treated plants, remained constant at 60 hours then decreased slightly at symptom collection.

Effect of treatments on yield. Yield, as measured by tomato fruit number and weight, was analyzed separately for each of three years as well as with the combined data. In 2004, immature fruit were harvested and no fruit were marketable. The PGPR compound was the only treatment that increased fruit number and weight significantly above the untreated control (P=0.0058, Table

Table 4.4. Effect of plant activators and copper on bacterial speck disease and tomato yield, 2004-2006.

Treatment	Lesion no.[ab]	Fruit no.[c]	Fruit wt[d]
Control	35.7 a	133.8 a	27.0 a
PGPR	29.2 b	136.8 a	27.9 a
ASM	9.4 c	121.5 a	25.2 a
ASM+PGPR	8.7 c	126.9 a	26.2 a
Copper	12.1 c	134.5 a	27.7 a

[a]Mean number of lesions on 20 leaflets per plot (averaged over three years).

[b]Means within a column sharing the same letters are not significantly different (P>0.05, Fisher's LSD).

[c]Mean number of fruit on 5 plants per plot.

[d]Mean weight (kg) of fruit from 5 plants per plot.

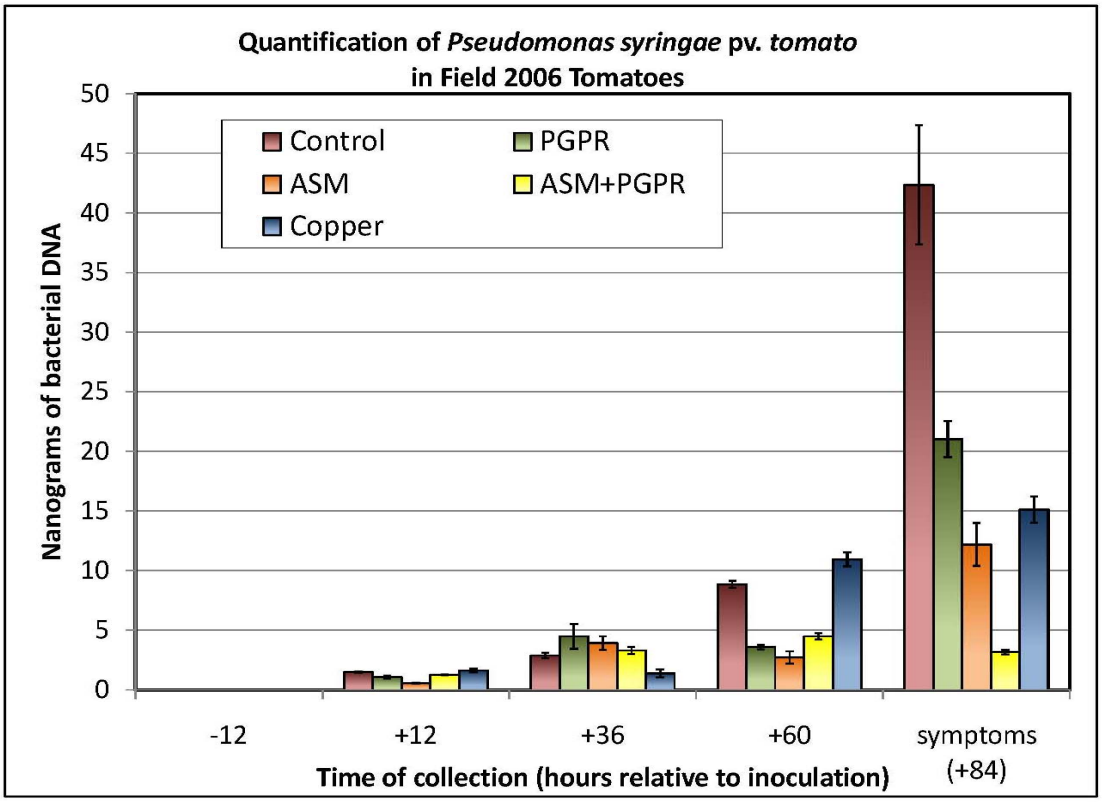


Figure 4.2. Quantification of *P. syringae* pv. *tomato* DNA present in 2006 tomato field samples. Red bars represent mean amount of pathogen DNA of untreated control plants, green bars denote PGPR-treated plants, orange bars indicate ASM-treated plants, yellow bars correspond to plants treated with both ASM and PGPR, and the copper treatment is signified by blue bars. Each bar represents the average amount of pathogen DNA (+/- SE) from three replicate plots. Plants were inoculated with *P. syringae* pv. *tomato* at time zero.

4.1). Neither ASM treatment significantly reduced yield relative to the untreated control.

Marketable yield was evaluated twice in 2005. No significant differences were observed between any of the treatments for either harvest for fruit number (*P*=0.8529, *P*=0.7921, respectively, Table 4.2) or weight (*P*=0.9953, *P*=0.9066, respectively).

Due to severe hail damage, no fruit were marketable in 2006 (Figure 4.3). No significant differences were observed for total yield among treatments for fruit number (*P*=0.3469, Table 4.5) or weight (*P*=0.4585). Differences for the extra large size class were observed as plants receiving the combination of ASM and the PGPR compound had significantly greater number (*P*=0.0010) and weight (*P*=0.0032) of fruit than the untreated control, though not statistically different from the copper treatment (*P*=0.0814, *P*=0.1624, respectively).

No significant differences were observed in tomato yield when data were combined over the three field seasons in fruit number (*P*=0.7756, Table 4.4) or weight (*P*=0.8465).

Treatment and signaling pathway activation. Quantification of defense gene expression was evaluated for three signaling pathway marker genes (relative to actin) via qRT-PCR for each of the five treatments. Complete results comparing inoculated versus noninoculated plants for each of the two seasons (2005 and 2006) are divided by signaling pathway and presented below (Figs. 4.2-4.7).

Figure 4.3. Hail damage and bacterial infection in field-grown tomatoes, 2006.

Table 4.5. Yield data 2006.

Treatment	Fruit no.[ab]	Fruit wt.[c]	XL Fruit no.	XL Fruit wt.
Control	62.5 a	21.2 a	13.3 b	8.4 b
PGPR	50.0 a	19.6 a	16.5 b	10.4 b
ASM	63.8 a	23.4 a	15.3 b	12.1 b
ASM+PGPR	74.8 a	27.3 a	24.7 a	15.0 a
Copper	70.5 a	25.2 a	19.2 ab	12.1 ab

[a]Mean number of fruit on 5 plants per plot.

[b]Means within a column sharing the same letters are not significantly different (P>0.05, Fisher's LSD)

[c]Mean weight (kg) of fruit from 5 plants per plot.

Acidic PR-1 expression, 2005. In 2005, all treatments demonstrated low levels of acidic *PR-1* expression prior to pathogen inoculation (Figure 4.4 A and B). Acidic *PR-1* was not activated in any of the noninoculated plots, with the exception of ASM treated plants (Figure 4.4 B). In untreated inoculated plants, activation of the salicylic signaling pathway increased to highest levels at symptom development (17 days after inoculation, 4.1 fold increase from -12 hours, Figure 4.4 A, maroon bars). Acidic *PR-1* expression gradually increased to peak expression levels in inoculated, PGPR-treated plants at symptom development (17 days after inoculation, 67.3 fold increase from -12 hours, Figure 4.4 A, green bars). Inoculated, ASM-treated plants, both with and without the PGPR compound, demonstrated a minimal response of the salicylic acid signaling pathway (Figure 4.4, orange and yellow bars). In contrast, uninoculated, ASM-treated plants had 2 fold higher acidic *PR-1* expression than all other treatments at -12 hours, with peak expression 24 hours after ASM application (Figure 4.4 B, orange bars). By 36 hours, expression levels were 9.6 times lower than at 12 hours, and levels remained low throughout the rest of the experiment. Copper-treated plants demonstrated minimal activation of the salicylic acid signaling pathway (Figure 4.4, blue bars). Both inoculated and noninoculated plots had fluctuating, low levels of acidic *PR-1* expression throughout the experiment.

Basic PR-1 expression, 2005. Activation of the ethylene signaling pathway in untreated inoculated plants was 4.5 fold lower 12 hours following inoculation, then increased to highest expression levels at symptom development (17 days after inoculation, 2.7 fold increase from -12 hours, Figure 4.5 A, maroon bars). Basic PR-1 induction of was highest at symptom

Figure 4.4. Expression pattern of the salicylic acid signaling pathway marker gene (acidic *PR-1*) for three experimental replicates in 2005 in relationship to inoculation with *P. syringae* pv. *tomato*. The top panel (A) represents plants inoculated with *P. syringae* pv. *tomato*, whereas the bottom panel (B) depicts noninoculated plots. Red bars represent mean expression level of untreated control plants, green bars denote PGPR-treated plants, orange bars indicate ASM-treated plants, yellow bars correspond to plants treated with both ASM and PGPR, and the copper treatment is signified by blue bars. Each bar represents the average induction (+/- SE) for three replicate plots of gene transcripts normalized to the housekeeping gene actin. Subsequently gene activation was normalized to the mean expression value prior to bacterial inoculation (-12 hour time point) for each treatment group. Plants were inoculated with *P. syringae* pv. *tomato* at time zero.

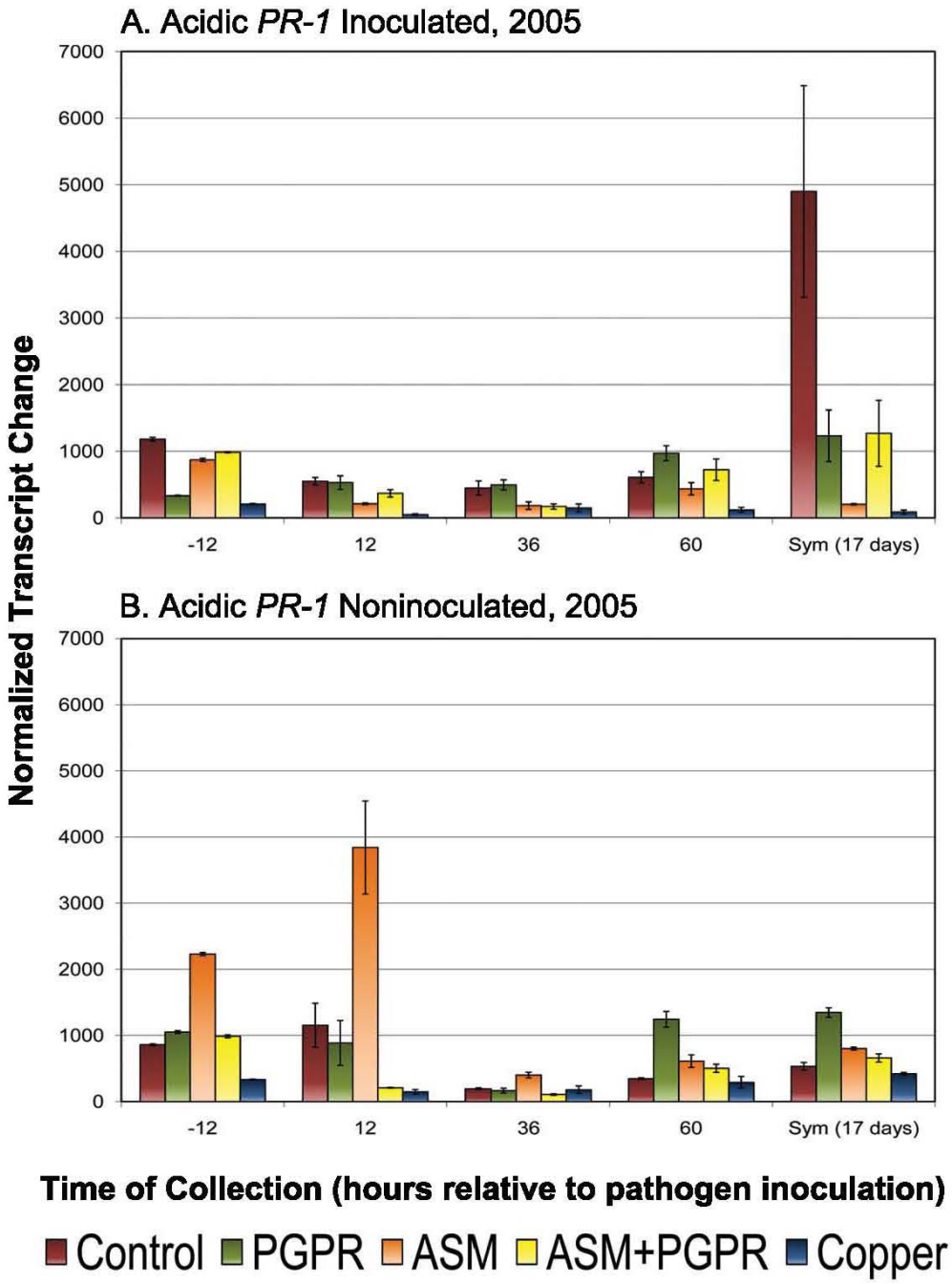
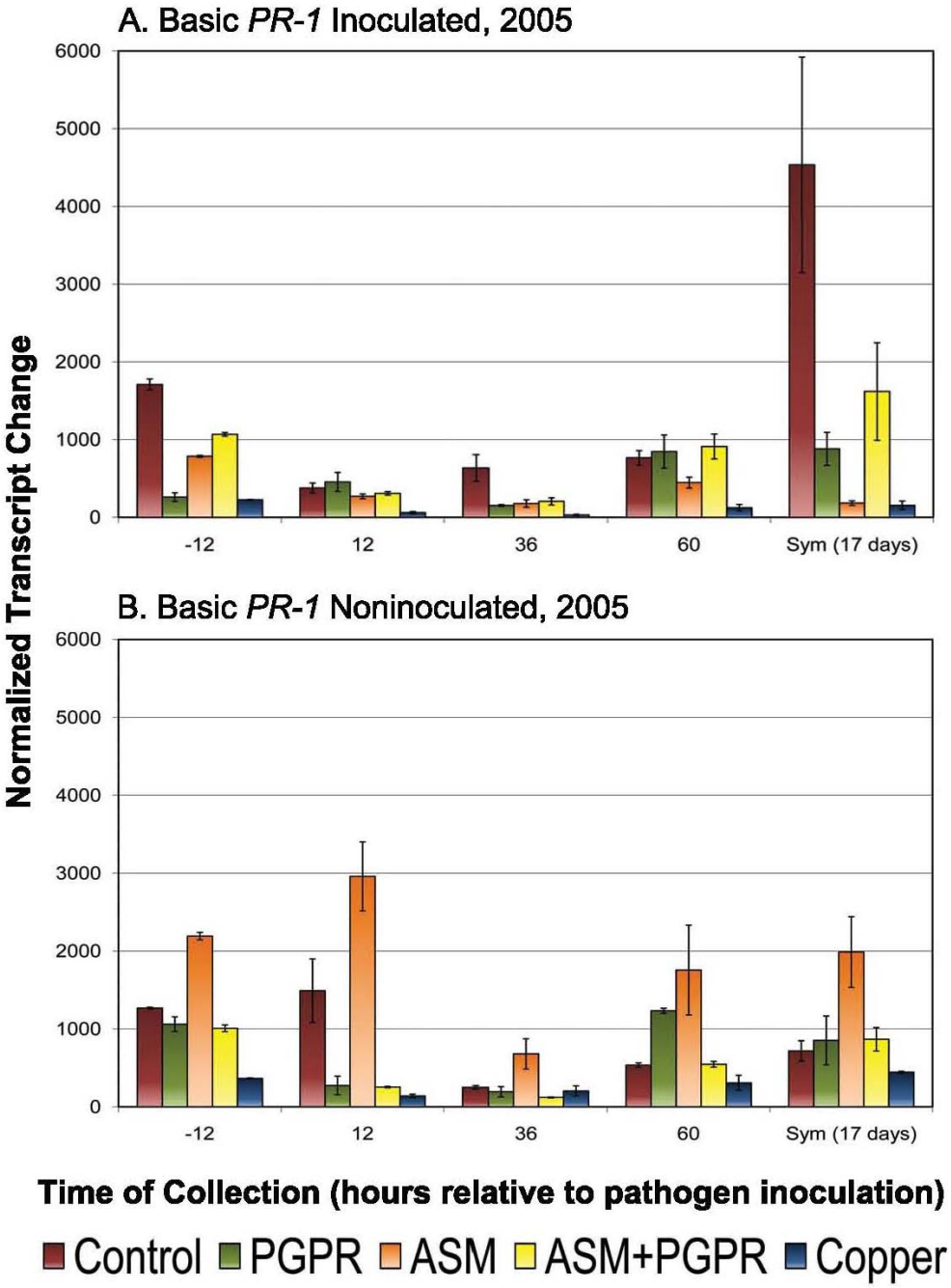

A. Acidic PR-1 Inoculated, 2005
B. Acidic PR-1 Noninoculated, 2005
Normalized Transcript Change
7000
6000
5000
4000
3000
2000
1000
0
-12
12
36
60
Sym (17 days)
Time of Collection (hours relative to pathogen inoculation)
Control
PGPR
ASM
ASM+PGPR
Copper

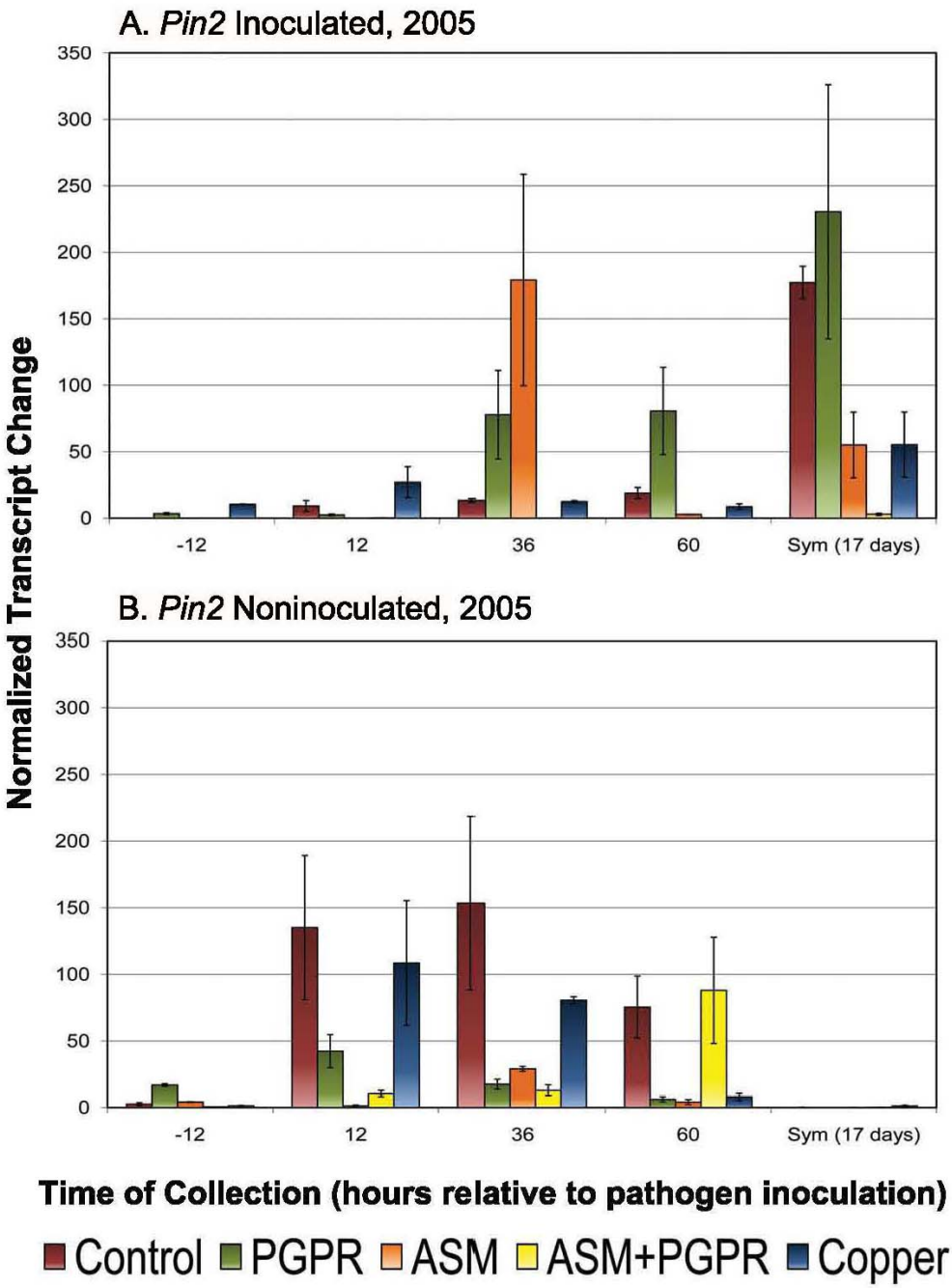
Figure 4.5. Expression pattern of the ethylene signaling pathway marker gene (basic PR-1) for three experimental replicates of the 2005 field season in relationship to inoculation with *P. syringae* pv. *tomato*. The top panel (A) represents plants inoculated with *P. syringae* pv. *tomato*, whereas the bottom panel (B) depicts noninoculated plots. Red bars represent mean expression level of untreated control plants, green bars denote PGPR-treated plants, orange bars indicate ASM-treated plants, yellow bars correspond to plants treated with both ASM and PGPR, and the copper treatment is signified by blue bars. Each bar represents the average induction (+/- SE) for three replicate plots of gene transcripts normalized to the housekeeping gene actin. Subsequently gene activation was normalized to the mean expression value prior to bacterial inoculation (-12 hour time point) for each treatment group. Plants were inoculated with *P. syringae* pv. *tomato* at time zero.


A. Basic PR-1 Inoculated, 2005
B. Basic PR-1 Noninoculated, 2005
Normalized Transcript Change
6000
5000
4000
3000
2000
1000
0
-12
12
36
60
Sym (17 days)
Time of Collection (hours relative to pathogen inoculation)
Control
PGPR
ASM
ASM+PGPR
Copper

development in PGPR-treated plants (17 days after inoculation, 3.4 fold increase from -12 hours, Figure 4.5 A, green bars). Prior to inoculation, basic *PR-1* levels were elevated in ASM-treated plants, with 2.8 fold higher expression observed in noninoculated plots (Figure 4.5, orange bars). Greatest induction of the ethylene signaling pathway was observed at -12 hours in inoculated plants, with lower, fluctuating expression levels throughout the remainder of the experiment (Figure 4.5 A). Treatment with ASM resulted in 2 fold higher basic *PR-1* activation at -12 hours relative to all other treatments in uninoculated plots with highest induction at 12 hours (Figure 4.5 B). Basic *PR-1* levels were elevated in ASM and PGPR-treated plants prior to inoculation, though the ethylene signaling pathway was not significantly induced in inoculated plants to levels above those observed at -12 hours (Figure 4.5 A, yellow bars). Similar to acidic *PR-1* expression, basic *PR-1* was activated at low levels in both inoculated and noninoculated plants treated with copper (Figure 4.5, blue bars).

Pin2 expression, 2005. The wound-induced jasmonic acid signaling pathway was activated at low levels throughout the course of the experiment (Figure 4.6). Several spikes of Pin2 expression were observed in noninoculated plants for each of the treatments (Figure 4.6 B). Following inoculation, *Pin2* expression increased to highest levels at symptom development in untreated (17 days after inoculation, 177 fold increase from -12 hours, Figure 4.6 A, maroon bars) and PGPR-treated plants (67.3 fold increase from -12 hours, Figure 4.6 A, green bars). Following inoculation, two spikes in *Pin2* induction were observed in ASM-treated plants (1236 and 380 fold increase from -12 hours, 36 hours and symptoms, respectively) whereas

Figure 4.6. Expression pattern of the jasmonic acid signaling pathway marker gene (*Pin2*) from three experimental replicates of the 2005 field season in relationship to inoculation with *P. syringae* pv. *tomato*. The top panel (A) represents plants inoculated with *P. syringae* pv. *tomato*, whereas the bottom panel (B) depicts noninoculated plots. Red bars represent mean expression level of untreated control plants, green bars denote PGPR-treated plants, orange bars indicate ASM-treated plants, yellow bars correspond to plants treated with both ASM and PGPR, and the copper treatment is signified by blue bars. Each bar represents the average induction (+/- SE) for three replicate plots of gene transcripts normalized to the housekeeping gene actin. Subsequently gene activation was normalized to the mean expression value prior to bacterial inoculation (-12 hour time point) for each treatment group. Plants were inoculated with *P. syringae* pv. *tomato* at time zero.


A. Pin2 Inoculated, 2005
B. Pin2 Noninoculated, 2005
Normalized Transcript Change
350
300
250
200
150
100
50
0
-12
12
36
60
Sym (17 days)
Time of Collection (hours relative to pathogen inoculation)
Control
PGPR
ASM
ASM+PGPR
Copper

little or no expression was observed at the other collection time points (Figure 4.6 A, orange bars). Expression of *Pin2* remained at very low levels in inoculated plots treated with the two plant activators throughout the course of the experiment (Figure 4.6 A, yellow bars). The jasmonic acid signaling pathway was activated a low levels prior to pathogen inoculation in copper-treated plants and increased to highest levels at symptom development (5.3 fold increase from -12 hours, Figure 4.6 A, blue bars).

Acidic PR-1 expression, 2006. Acidic *PR-1* was expressed at low levels in all plots prior to pathogen inoculation (Figure 4.7). Activation of the salicylic acid signaling pathway gradually increased to highest levels at symptom development in untreated plants (84 hours after inoculation, 6.1 fold increase from -12 hours, Figure 4.7 A, maroon bars). Acidic *PR-1* was expressed at low levels in PGPR-treated plants prior to pathogen inoculation, increasing to highest levels at 60 hours (3.4 fold increase from -12 hours, Figure 4.7 A, green bars) and remaining elevated through symptom development. In ASM and copper-treated plants, induction of the salicylic acid defenseresponse pathway increased to highest levels at 60 hours and rapidly declined by symptom development (84 hours after inoculation, 22 and 16.9 fold increase from -12 hours, Figure 4.7 A, orange and blue bars, respectively). Acidic *PR-1* expression gradually increased following inoculation in ASM and PGPR-treated plants to highest levels at 60 hours (2.5 fold increase from -12 hours, Figure 4.7 A, yellow bars) and dropped significantly at symptom development.

Basic PR-1 expression, 2006. Basic *PR-1* was expressed at low levels prior to inoculation in all plots (Figure 4.8). Each noninoculated treatment

Figure 4.7. Expression pattern of the salicylic acid signaling pathway marker gene (acidic *PR-1*) from three experimental replicates of 2006 field-grown tomatoes in relationship to inoculation with *P. syringae* pv. *tomato*. The top panel (A) represents plants inoculated with *P. syringae* pv. *tomato*, whereas the bottom panel (B) depicts noninoculated plots. Red bars represent mean expression level of untreated control plants, green bars denote PGPR-treated plants, orange bars indicate ASM-treated plants, yellow bars correspond to plants treated with both ASM and PGPR, and the copper treatment is signified by blue bars. Each bar represents the average induction (+/- SE) for three replicate plots of gene transcripts normalized to the housekeeping gene actin. Subsequently gene activation was normalized to the mean expression value prior to bacterial inoculation (-12 hour time point) for each treatment group. Plants were inoculated with *P. syringae* pv. *tomato* at time zero.

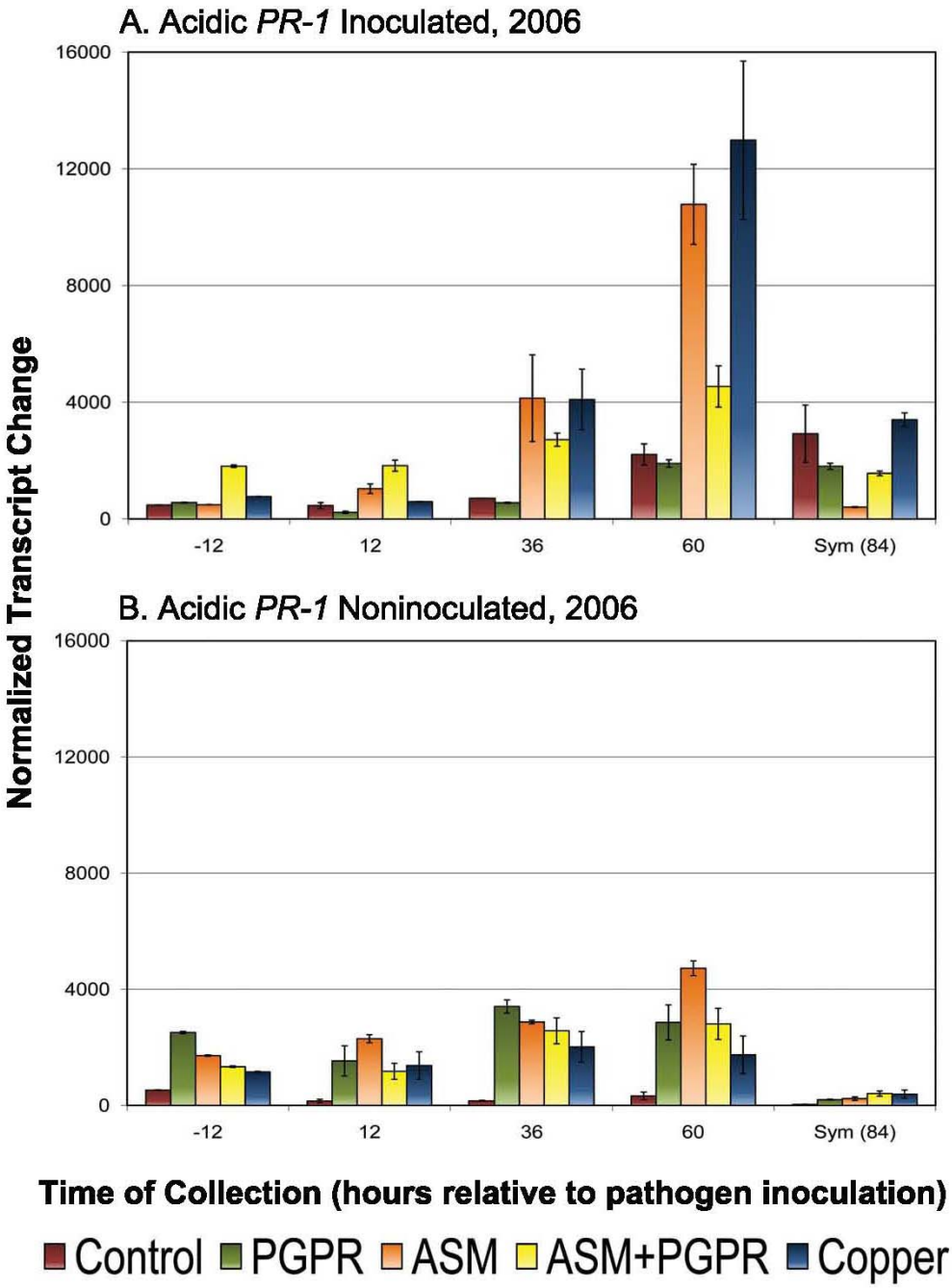
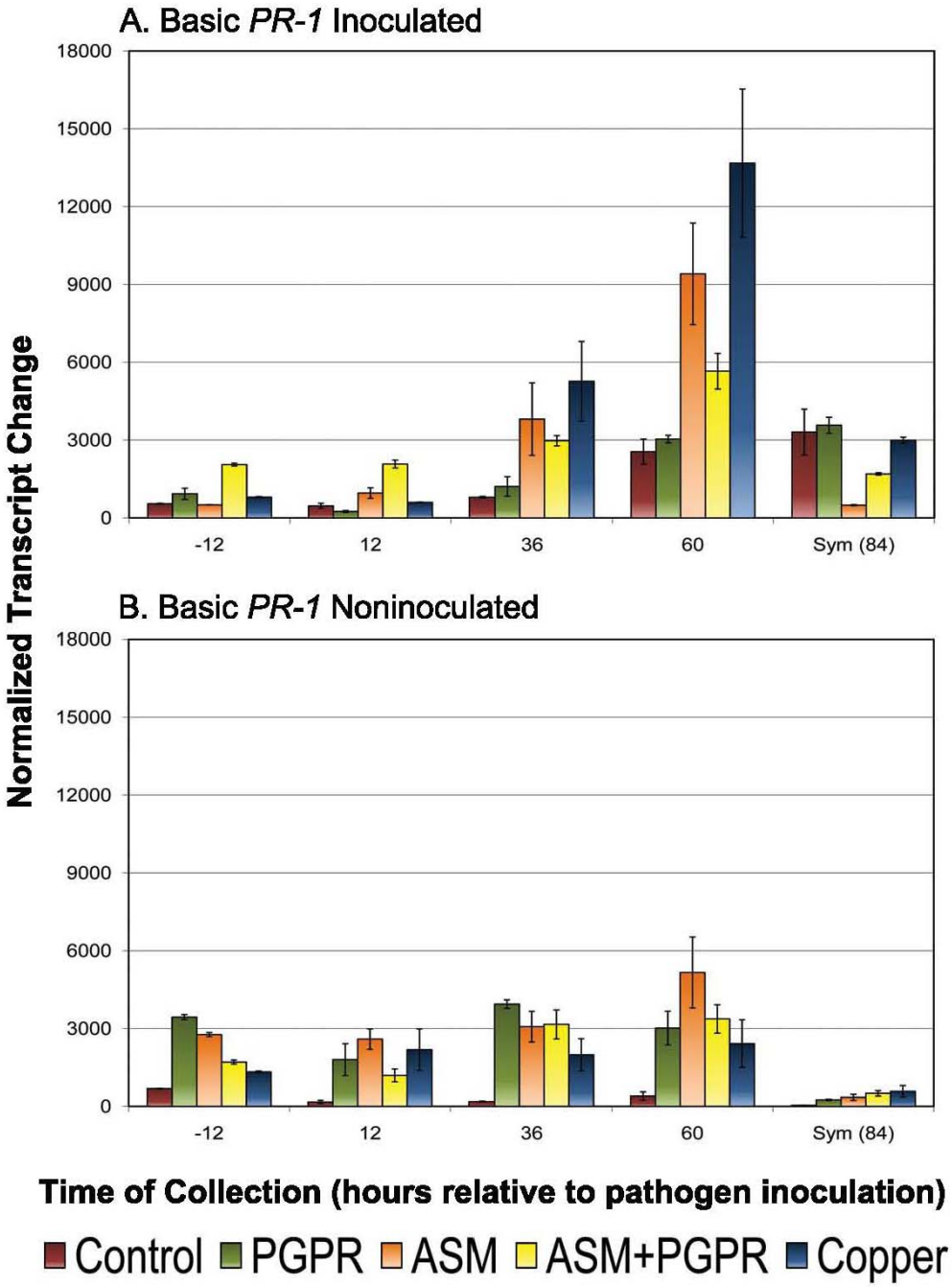

A. Acidic PR-1 Inoculated, 2006
16000
12000
8000
4000
0
-12
12
36
60
Sym (84)
B. Acidic PR-1 Noninoculated, 2006
16000
12000
8000
4000
0
-12
12
36
60
Sym (84)
Normalized Transcript Change
Time of Collection (hours relative to pathogen inoculation)
Control
PGPR
ASM
ASM+PGPR
Copper

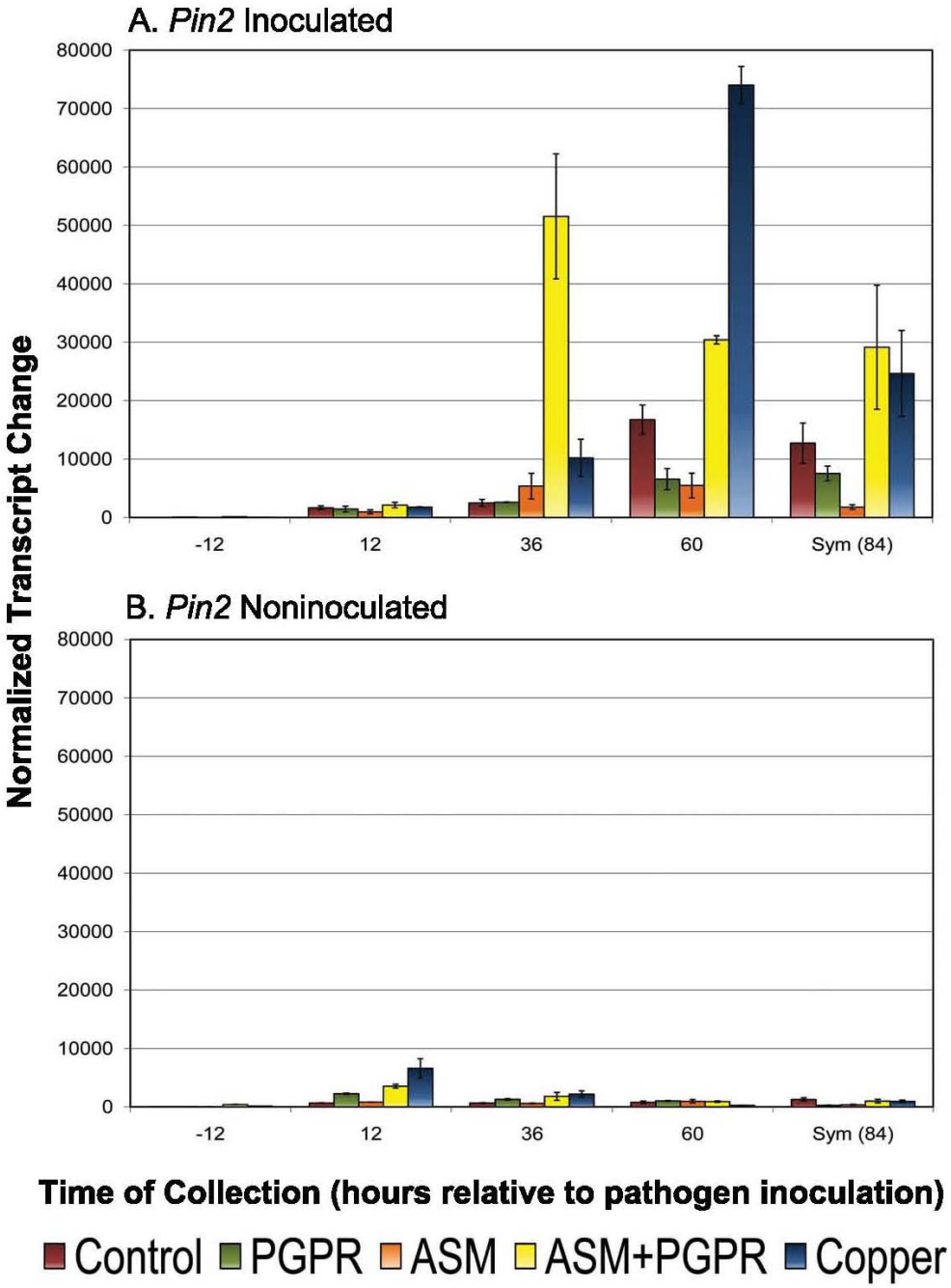
Figure 4.8. Expression pattern of the ethylene signaling pathway marker gene (basic *PR-1*) from 2006 field tomatoes with three experimental replicates in relationship to inoculation with *P. syringae* pv. *tomato*. The top panel (A) represents plants inoculated with *P. syringae* pv. *tomato*, whereas the bottom panel (B) depicts noninoculated plots. Red bars represent mean expression level of untreated control plants, green bars denote PGPR-treated plants, orange bars indicate ASM-treated plants, yellow bars correspond to plants treated with both ASM and PGPR, and the copper treatment is signified by blue bars. Each bar represents the average induction (+/- SE) for three replicate plots of gene transcripts normalized to the housekeeping gene actin. Subsequently gene activation was normalized to the mean expression value prior to bacterial inoculation (-12 hour time point) for each treatment group. Plants were inoculated with *P. syringae* pv. *tomato* at time zero.


A. Basic PR-1 Inoculated
B. Basic PR-1 Noninoculated
Normalized Transcript Change
18000
15000
12000
9000
6000
3000
0
-12
12
36
60
Sym (84)
Time of Collection (hours relative to pathogen inoculation)
Control
PGPR
ASM
ASM+PGPR
Copper

demonstrated consistent basic *PR-1* expression from -12 to 60 hours, with expression levels decreasing at the 84 hour collection (Figure 4.8 B). Activation of the ethylene signaling pathway in untreated and PGPR-treated inoculated plants increased to highest expression levels at symptom development (84 hours after inoculation, 6.1 and 3.8 fold increase from -12 hours, Figure 4.8 A, maroon and green bars, respectively). In copper and both ASM-treated plots, basic *PR-1* was expressed at highest levels at 60 hours and rapidly declined by symptom development (84 hours after inoculation). At peak expression levels, ASM treated plants exhibited an 18.6 fold increase (Figure 4.8 A, orange bars), ASM and PGPR treated plants demonstrated a 2.6 fold induction (Figure 4.8 A, yellow bars) and copper-treated plants responded with a 17.1 fold increase from -12 hours (Figure 4.8 A, blue bars).

Pin2 expression, 2006. The jasmonic acid signaling pathway was not activated prior to pathogen inoculation in any treatment plot (Figure 4.9 A and B). The -12 collection timepoint occurred prior to a hail storm. Following the storm and subsequent inoculation with *P. syringae* pv. *tomato*, *Pin2* expression in untreated plants increased 109.5 and 14.8 fold in inoculated and noninoculated plants, respectively (12 hours, Figure 4.9 A and B, maroon bars). Highest levels of *Pin2* expression in untreated inoculated plants were observed at 60 hours (1096 fold increase from -12 hours), though levels remained elevated at symptom development (84 hours post inoculation, 833 fold increase from -12 hours, Figure 4.9 A). PGPR-treated plants responded at 12 hours with increased *Pin2* expression of 17.5 and 37 fold in inoculated and noninoculated plants, respectively (Figure 4.9 A and B, green bars). Greatest *Pin2* expression in inoculated PGPR-treated plants was observed at

Figure 4.9. Expression pattern of the jasmonic acid signaling pathway marker gene (*Pin2*) with three experimental replicates of 2006 field-grown tomatoes in relationship to inoculation with *P. syringae* pv. *tomato*. The top panel (A) represents plants inoculated with *P. syringae* pv. *tomato*, whereas the bottom panel (B) depicts noninoculated plots. Red bars represent mean expression level of untreated control plants, green bars denote PGPR-treated plants, orange bars indicate ASM-treated plants, yellow bars correspond to plants treated with both ASM and PGPR, and the copper treatment is signified by blue bars. Each bar represents the average induction (+/- SE) for three replicate plots of gene transcripts normalized to the housekeeping gene actin. Subsequently gene activation was normalized to the mean expression value prior to bacterial inoculation (-12 hour time point) for each treatment group. Plants were inoculated with *P. syringae* pv. *tomato* at time zero.


A. *Pin2* Inoculated
B. *Pin2* Noninoculated
Normalized Transcript Change
80000
70000
60000
50000
40000
30000
20000
10000
0
-12
12
36
60
Sym (84)
Time of Collection (hours relative to pathogen inoculation)
Control
PGPR
ASM
ASM+PGPR
Copper

symptom development (84 hours post inoculation, 91.7 fold increase from -12 hours, Figure 4.9 A). Highest *Pin2* expression in ASM-treated inoculated plots was observed at 36 and 60 hours post inoculation, with levels declining by symptom development (84 hours, Figure 4.9 A, orange bars). *Pin2* was activated from -12 to 12 hours (21.2 fold increase from -12 hours) in ASM-treated noninoculated plants and levels declined over the remainder of the experiment (Figure 4.9 B). Following the storm and inoculation, *Pin2* expression increased in ASM and PGPR-treated plots 17.5 and 9.1 fold from -12 hours in inoculated and noninoculated plants, respectively (12 hours, Figure 4.9 A and B, yellow bars). Highest *Pin2* expression in inoculated plants was observed at 36 hours post inoculation (423 fold increase from -12 hours, Figure 4.9 A) with levels remaining elevated for the remainder of the experiment (239-250 fold increase from -12 hours). The jasmonic acid signaling pathway was induced in copper-treated plants to highest levels at 60 hours (1263 fold increase from -12 hours, Figure 4.9 A, blue bars) and declined by symptom development (84 hours post inoculation, 420.6 fold induction from -12 hours). *Pin2* was induced in noninoculated copper-treated plants following the hail storm (12 hours, 56.2 fold induction from -12 hours, Figure 4.9 B).

DISCUSSION

Findings of this study indicate that plant activators reduce severity of bacterial speck disease and do not negatively affect yield of field-grown tomatoes. In two of the three years, all treatments significantly lowered the number of bacterial speck lesions relative to the untreated control.

The PGPR compound reduced bacterial speck symptoms relative to the untreated control in two of the three experiments and under low disease pressure (2005) controlled *P. syringae* pv. *tomato* as well as ASM and copper (Tables 4.1-4.3). In contrast, copper and ASM (both alone and in combination with the PGPR compound) effectively reduced bacterial speck symptoms all three years (Tables 4.1-4.3). Combination of the two plant activators resulted in lower, but not statistically significant, disease severity than ASM alone in 2004 and 2005. Evaluating disease control across the three year study indicates that PGPR significantly reduced symptoms relative to the untreated control, but not to the same degree of copper and both ASM treatments (Table 4.4).

Quantification of *P. syringae* pv. *tomato* was examined to determine if treatments reduced the amount of bacterial growth. The purpose of this experiment was to further understand the correlation between bacterial growth and visible disease symptoms. Previous research has shown that the two are not always directly related (Brouwer et al. 2003). As expected, untreated control plants exhibited exponential growth of *P. syringae* pv. *tomato* over the course of this experiment (Figure 4.2). All treatments negatively impacted bacterial growth, providing valuable information as it is not fully understood how plant activators interact with target plants and pathogens (McSpadden Gardener and Fravel 2002). The untreated control had both the highest level of bacterial DNA at 84 hours after inoculation, and also the significantly largest number of lesions at 4 days (Table 4.3). The same is true for the PGPR-treated plants, which had significantly fewer lesions, and significantly less bacterial DNA. No significant differences were found between the number of

lesions for the other three treatments. However, *P. syringae* pv. *tomato* did not appear to grow on plants treated with both ASM and the PGPR compound as the amount of bacterial DNA increased only slightly by 60 hours and decreased at symptom development (Figure 4.2, yellow bars). In the PGPR, ASM and copper-treatments, amount of bacterial DNA at symptom development was at least half that of the untreated control, indicating that each treatment detrimentally affected pathogen growth. It is unknown why ASM and PGPR reduce bacterial populations, however there was also a large increase in *Pin2* gene expression in this treatment at the 36 hour timepoint (Figure 4.9). It is possible that this large increase in the jasmonic acid signaling pathway may be involved in bacterial control.

In 2004, the PGPR compound was the only treatment that resulted in an increase in fruit number and weight, though this yield boost was not seen it was combined with ASM (Table 4.1). Low disease pressure and exceptional growing conditions in 2005 enabled all treatments to produce large yields. In 2006, no differences were observed in total yield, though the combination of plant activators resulted in significantly more extra large fruit (Table 4.6). Copper-treated plants also had elevated, though not statistically significant, levels of extra large tomatoes. Despite having disease control equivalent to copper and ASM and PGPR-treated plots, an increase in extra large tomatoes was not observed in plants treated with ASM.

In contrast to results observed in greenhouse studies (Chapter 2), treatment with ASM, alone or in combination with the PGPR compound, did not result in higher acidic or basic *PR-1* expression relative to the untreated control prior to inoculation. Only ASM-treated noninoculated plots in 2005

exhibited elevated acidic and basic *PR-1* expression at the -12 and 12 hour time points, though it is difficult to draw significance from this as expression at the -12 time points should have been comparable between noninoculated and inoculated plots. Under low disease pressure in 2005, both ASM treatments failed to show any significant activation of the salicylic acid or ethylene signaling pathway in response to inoculation. In 2006, both noninoculated and inoculated plants demonstrated increasing acidic and basic *PR-1* activation from -12 to 60 hours, with expression dropping dramatically at symptom development (84 hours, Figs. 4.7 and 4.8). In ASM-treated plots, fold induction of the salicylic acid and ethylene signaling pathways was significantly higher in inoculated plots compared with uninoculated (22 versus 2.8 fold for salicylic acid and 18.6 versus 1.9 fold for ethylene, Figs. 4.7 and 4.8). ASM and PGPR-treated plots also demonstrated a similar increase in acidic and basic *PR-1* expression following inoculation, though possible negative crosstalk occurs as fold increase was significantly lower than observed with ASM-treated plants. Interestingly, this decrease in defense pathway activation appears to have no significant effect on disease control (Figs. 4.7 and 4.8, Table 4.3).

Activation of the jasmonic acid signaling pathway was minimal in 2005 (Figure 4.6). Taken into account with the low levels of disease, it is likely that the observed spikes in activation from -12 to 60 hours are due to plant injury in the field (for example insect herbivory). While no activation of *Pin2* was observed in noninoculated plants at the symptomatic collection time (17 days), levels were elevated in all inoculated treatments, with the exception of ASM and PGPR-treated plants. Unlike results seen in greenhouse studies,

activation of the wound-induced signaling pathway did not correspond to the number of *P. syringae* pv. *tomato* lesions per leaflet in these field trials.

In 2006, no induction of the jasmonic acid pathway was observed prior to inoculation and expression levels increased in all treatments following a hail storm and subsequent pathogen inoculation in both inoculated and noninoculated plots (Figure 4.9). Compared to activation at later time points, the destruction from the hail storm activated *Pin2* at low levels. However, by looking at the normalized transcript change values in 2005 (the highest value is 350) and 2006 (the highest value is 80,000, Figs. 4.6 and 4.9), it is clear that the hail storm drastically increased expression. Following inoculation, activation of *Pin2* in untreated control, PGPR and ASM-treated plants reflected disease severity. In contrast, copper and ASM and PGPR-treated plants exhibited dramatic (several hundred fold) increases in expression between 36 hours and symptom development. Significant wounding from the hail storm could have impacted signaling in plots treated with both plant activators. Interactions between pathogen, copper and hail damage could have resulted in the striking increase in *Pin2* expression, as this elevated activation was not observed in noninoculated plants (Figure 4.9).

PGPR-treated plants did not appear to modify gene expression prior to inoculation in the field. There was no evidence of priming in PGPR-treated plants as neither acidic nor basic *PR-1* was induced earlier, or to a greater degree than untreated control plants in either year. The *Bacillus* spp. used in this study have been found in other systems to induce acidic *PR-1*, lipoxygenase, phenylalanine ammonia-lyase (PAL), and 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), and to activate ISR via salicylic and

jasmonic acid signaling pathways (Ahn et al. 2002; Ryu et al. 2007). Other *Bacillus* species have been found to induce β-glucanase, peroxidase and chitinase and increase levels of salicylic acid (Bargabus et al. 2003, 2004; Zhang et al. 2002). It is possible that PGPR-induced gene expression changes were too small to detect above background fluctuation in the field, as the compound provided significant reduction of disease symptoms in both 2005 and 2006. Utilization of other marker genes associated with ISR-induction could reveal expression changes.

While the PGPR compound provided some control of the pathogen, it was not to commercially acceptable levels. Use of both plant activators together provided comparable disease control to ASM alone, and resulted in yield increases in one of three years. All compounds used in this study modified pathogen growth in the field, though this result did not correlate with disease symptoms. Synthesis of these data indicate that the PGPR-containing compound may provide a boost in yield, ASM provides a viable control option for *P. syringae* pv. *tomato* in New York, and that there is no negative effect on disease control if both compounds are applied to the same plants. Further studies are necessary to determine if the application of ASM modifies a yield increase due to PGPR (as seen in 2004), or if both compounds together may aid in reducing bacterial populations under severe epidemics (as seen in 2006).

REFERENCES

Ahn, I. P., Park, K., and Kim, C. H. 2002. Rhizobacteria-induced resistance perturbs viral disease progress and triggers defense-related gene expression. *Molecules and Cells* 13 (2):302-308.

Alexander, S.A., Kim, S.H., and Waldenmaier, C.M. 1999. First report of copper-tolerant *Pseudomonas syringae* pv. *tomato* in Virginia. *Plant Disease* 83 (10):964.

Anith, K. N., Momol, M. T., Kloepper, J. W., Marois, J. J., Olson, S. M., and Jones, J. B. 2004. Efficacy of plant growth-promoting rhizobacteria, acibenzolar-*S*-methyl, and soil amendment for integrated management of bacterial wilt on tomato. *Plant Disease* 88 (6):669-673.

Bargabus, R. L., Zidack, N. K., Sherwood, J. E., and Jacobsen, B. J. 2003. Oxidative burst elicited by *Bacillus mycoides* isolate Bac J, a biological control agent, occurs independently of hypersensitive cell death in sugar beet. *Molecular Plant-Microbe Interactions* 16 (12):1145-53.

Bargabus, R. L., Zidack, N. K., Sherwood, J. E., and Jacobsen, B. J. 2004. Screening for the identification of potential biological control agents that induce systemic acquired resistance in sugar beet. *Biological Control* 30 (2):342-350.

Beaubois, E., Girard, S., Lallechere, S., Davies, E., Paladian, F., Bonnet, P., Ledoigt, G., and Vian, A. 2007. Intercellular communication in plants: Evidence for two rapidly transmitted systemic signals generated in response to electromagnetic field stimulation in tomato. *Plant Cell and Environment* 30 (7):834-844.

Bender, C. L., and Cooksey, D. A. 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato* - conjugative transfer and role in copper resistance. *Journal of Bacteriology* 165 (2):534-541.

Bostock, R. M. 2005. Signal crosstalk and induced resistance: straddling the line between cost and benefit. *Annual Review of Phytopathology* 43:545-580.

Brouwer, M., Lievens, B., Van Hemelrijck, W., Van den Ackerveken, G., Cammue, B.P.A, and Thomma, B.P.H.J. 2003. Quantification of disease progression of several microbial pathogens on *Arabidopsis thaliana* using real-time fluorescence PCR. *Fems Microbiology Letters* 228:241-248.

Buonaurio, R., Stravato, V. M., and Capelli, C. 1996. Occurrence of Pseudomonas syringae pv tomato race 1 in Italy on Pto gene-bearing

tomato plants. *Journal of Phytopathology-Phytopathologische Zeitschrift* 144 (9-10):437-440.

Cuppels, D. A., and Elmhirst, J. 1999. Disease development and changes in the natural *Pseudomonas syringae* pv. *tomato* populations on field tomato plants. *Plant Disease* 83 (8):759-764.

Dietrich, R., Ploss, K., and Heil, M. 2005. Growth responses and fitness costs after induction of pathogen resistance depend on environmental conditions. *Plant Cell and Environment* 28 (2):211-222.

Ding, J. Y., Jia, J. W., Yang, L. T., Wen, H. B., Zhang, C. M., Liu, W. X., and Zhang, D. B. 2004. Validation of a rice specific gene, sucrose phosphate synthase, used as the endogenous reference gene for qualitative and real-time quantitative PCR detection of transgenes. *Journal of Agricultural and Food Chemistry* 52 (11):3372-3377.

Domenech, J., Reddy, M. S., Kloepper, J. W., Ramos, B., and Gutierrez-Manero, J. 2006. Combined application of the biological product LS213 with *Bacillus*, *Pseudomonas* or *Chryseobacterium* for growth promotion and biological control of soil-borne diseases in pepper and tomato. *Biocontrol* 51 (2):245-258.

Durrant, W. E., and Dong, X. 2004. Systemic acquired resistance. *Annual Review of Phytopathology* 42:185-209.

Goode, M.J., and Sasser, M. 1980. Prevention - The key to controlling bacterial spot and bacterial speck of tomato. *Plant Disease* 64:831-834.

Guo, J. H., Qi, H. Y., Guo, Y. H., Ge, H. L., Gong, L. Y., Zhang, L. X., and Sun, P. H. 2004. Biocontrol of tomato wilt by plant growth-promoting rhizobacteria. *Biological Control* 29 (1):66-72.

He, Z. L. L., Yang, X. E., and Stoffella, P. J. 2005. Trace elements in agroecosystems and impacts on the environment. *Journal of Trace Elements in Medicine and Biology* 19 (2-3):125-140.

Jetiyanon, K., Fowler, W. D., and Kloepper, J. W. 2003. Broad-spectrum protection against several pathogens by PGPR mixtures under field conditions in Thailand. *Plant Disease* 87 (11):1390-1394.

Jones, J.B., Stall, R.E., and Zitter, T.A., eds. 1991. *Compendium of Tomato Diseases*. St. Paul: APS Press.

Kloepper, J. W., Ryu, C. M., and Zhang, S. A. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94 (11):1259-1266.

Kloepper, J.W., Reddy, M.S., Rodriguez-Kabana, R., Kenney, D.S., Kokalis-Burelle, N., and Martinez-Ochoa, N. 2004. Application for rhizobacteria in transplant production and yield enhancement. *Acta Horticulturae* 631:217-229.

Lawton, M. B., and Macneill, B. H. 1986. Occurrence of race 1 of *Pseudomonas syringae* pv. *tomato* on field tomato in southwestern Ontario. *Canadian Journal of Plant Pathology* 8 (1):85-88.

Louws, F. J., Wilson, M., Campbell, H. L., Cuppels, D. A., Jones, J. B., Shoemaker, P. B., Sahin, F., and Miller, S. A. 2001. Field control of bacterial spot and bacterial speck of tomato using a plant activator. *Plant Disease* 85 (5):481-488.

McSpadden Gardener, B.B., and Fravel, D.R. 2002. Biological control of plant pathogens: Research, commercialization, and application in the USA. *Plant Health Progress*.

Mittapalli, O., Shukle, R.H., Sardesai, N., Giovanini, M.P., and Williams, C.E. 2006. Expression patterns of antibacterial genes in the Hessian fly. *Journal of Insect Physiology* 52:1143-1152.

Murphy, J. F., Reddy, M. S., Ryu, C. M., Kloepper, J. W., and Li, R. H. 2003. Rhizobacteria-mediated growth promotion of tomato leads to protection against Cucumber mosaic virus. *Phytopathology* 93 (10):1301-1307.

Pedley, K. F., and Martin, G. B. 2003. Molecular basis of Pto-mediated resistance to bacterial speck disease in tomato. *Annual Review of Phytopathology* 41:215-243.

Persello-Cartieaux, F., Nussaume, L., and Robaglia, C. 2003. Tales from the underground: molecular plant-rhizobacteria interactions. *Plant Cell and Environment* 26 (2):189-199.

Reiners, S., and Petzoldt, C., eds. 2007. *The integrated crop and pest management guidelines for vegetables*. Ithaca: Cornell University.

Ryu, C. M., Murphy, J. F., Reddy, M. S., and Kloepper, J. W. 2007. A two-strain mixture of rhizobacteria elicits induction of systemic resistance against *Pseudomonas syringae* and Cucumber mosaic virus coupled to promotion of plant growth on Arabidopsis thaliana. *Journal of Microbiology and Biotechnology* 17 (2):280-286.

Schaad, N.W., Jones, J.B., and Chun, W., eds. 2001. *Laboratory guide for identification of plant pathogenic bacteria*. Third ed. St. Paul: APS Press.

Scheck, H. J., Pscheidt, J. W., and Moore, L. W. 1996. Copper and streptomycin resistance in strains of *Pseudomonas syringae* from Pacific Northwest nurseries. *Plant Disease* 80 (9):1034-1039.

Silva, V.L., and Lopes, C.A. 1995. *Pseudomonas syringae* pv. *tomato* resistant to copper in copper-sprayed tomato fields. *Fitopatologia Brasileira* 20:85-89.

Smart, C.D., Borsick, M.A., and Lange, H.W. 2005. Producing disease-free transplants. Proceedings of the Empire State Fruit and Vegetable Expo, Syracuse.

Sticher, L., MauchMani, B., and Metraux, J. P. 1997. Systemic acquired resistance. *Annual Review of Phytopathology* 35:235-270.

Stout, M. J., Brovont, R. A., and Duffey, S. S. 1998. Effect of nitrogen availability on expression of constitutive and inducible chemical defenses in tomato, Lycopersicon esculentum. *Journal of Chemical Ecology* 24 (6):945-963.

Vallad, G. E., and Goodman, R. M. 2004. Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop Science* 44 (6):1920-1934.

van Loon, L. C., Bakker, P. A., and Pieterse, C. M. 1998. Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology* 36:453-83.

Yun, J. J., Heisler, L. E., Hwang, I. I. L., Wilkins, O., Lau, S. K., Hyrcza, M., Jayabalasingham, B., Jin, J., McLaurin, J., Tsao, M. S., and Der, S. D. 2006. Genomic DNA functions as a universal external standard in quantitative real-time PCR. *Nucleic Acids Research* 34 (12):E85-E85.

Yunis, H., Bashan, Y., and Henis, Y. 1980. Weather dependence, yield losses, and control of bacterial speck of tomato caused by *Pseudomonas syringae*. *Plant Disease* 64:937-939.

Zaccardelli, M., Spasiano, A., Bazzi, C., and Merighi, M. 2005. Identification and in planta detection of Pseudomonas syringae pv. tomato using PCR amplification of hrpZ(Pst). *European Journal of Plant Pathology* 111 (1):85-90.

Zhang, S., Reddy, M. S., and Kloepper, J. W. 2002. Development of assays for assessing induced systemic resistance by plant growth-promoting rhizobacteria against blue mold of tobacco. *Biological Control* 23 (1):79-86.

CHAPTER FIVE

CONCLUSIONS AND PERSPECTIVES ON PLANT ACTIVATORS AND BACTERIAL SPECK CONTROL IN TOMATO

The concept of activating plant defenses against pathogen and insect pests is appealing, but has not been implemented effectively in the field. Though induced resistance has been investigated in the laboratory for decades (Chester 1933, 1933; Kuc 1982), it is not fully known how plant activators interact with target plants and pathogens (McSpadden Gardener and Fravel 2002). For plant activators to be incorporated into a pest management scheme in the field, greater understanding of the molecular mechanisms of induced signaling is needed.

Currently, copper-based bactericides and cultural methods are used to control *Pseudomonas syringae* pv. *tomato*, causal agent of bacterial speck disease of tomato (Reiners and Petzoldt 2007; Smart et al. 2005). Acibenzolar-*S*-methyl (ASM), a systemic acquired resistance (SAR)-inducing compound, is commercially available and recommended for control of tomato bacterial diseases, though not widely used by growers in New York due to inconsistent performance in the field. Plant growth-promoting rhizobacteria are thought to increase plant health and vigor while controlling pests via induced systemic resistance (ISR). Potential cross-talk between signaling pathways used by these plant activators could lead to enhanced disease control and yield or negatively impact the plant host. This study examined the timing and duration of plant responses to activators and their effect on disease control and yield to determine how to best integrate them into tomato production in New York State.

Incorporation of plant activators into a greenhouse transplant system was investigated in Chapter 2. Under high pathogen pressure, both ASM and the PGPR compound reduced disease incidence and severity, though ASM provided significantly better disease control. Combined use of both activators controlled *P. syringae* pv. *tomato* as well as or better than ASM alone, indicating negative cross-talk did not occur between signaling pathways.

Induction of the wound-induced jasmonic acid signaling pathway directly correlated to amount of disease in greenhouse-grown plants (Chapter 2). This is not surprising, as the bacterial speck lesions are known to induce this pathway. Activation of was significantly less is ASM-treated plants, reflecting the lower levels of disease observed. Plants treated with ASM, alone or in combination with the PGPR compound, exhibited elevated levels of salicylic acid and ethylene signaling, with greater induction observed following pathogen inoculation. The PGPR compound inconsistently primed plants to respond faster and to a greater degree, though wound-induced signaling was greater than ASM-treated plants (reflecting the lower level of disease control). Increasing activation of the ethylene and salicylic acid signaling pathways was observed in untreated control and PGPR-treated plants in response to inoculation with *P. syringae* pv. *tomato*.

Data from this study indicate that incorporation of ASM into greenhouse transplant production could be useful for bacterial speck control. While the PGPR compound failed to provide sufficient pathogen control, it did not prevent ASM from activating signaling pathways and controlling disease. The PGPR used in this study has been shown to have growth-promoting effects when implemented in transplant and field production (Kloepper, Ryu, and

Zhang 2004; Kloepper, Reddy et al. 2004). Results imply that use of the plant activators together may provide the disease control benefits of ASM while boosting plant yield, and this was further investigated in Chapter 4.

Chapter three investigated tomato signaling pathway activation by ASM under field conditions in the absence of the pathogen. Responses of three tomato cultivars were followed over the course of two ASM applications. This study provided valuable information on defense signaling under field conditions as previous work has primarily focused on plants grown in the greenhouse or growth chamber. While degree of activation varied between cultivars, greater response of the salicylic acid and ethylene pathways was observed following the second ASM application. Peak expression of both pathways occurred one to two days after ASM treatment and activation returned to baseline levels by seven days. As expected, ASM did not induce the jasmonic acid signaling pathway.

Results of this study (Chapter 3) provide information that will facilitate future studies to determine whether ASM has a larger impact on disease control in cultivars that respond with higher levels of defense gene activation following application. In addition, it is necessary to monitor marker gene expression following plant activator application and pathogen inoculation to ascertain whether plant defenses are re-activated after pathogen attacks.

A comprehensive, three year analysis of plant activators and copper for field control of *P. syringae* pv. *tomato* composed the final portion of this study (Chapter 4). This experiment synthesized information from Chapters 2 and 3 to evaluate whether plant activators could be effectively incorporated into a disease management strategy in New York.

Similar to results of the greenhouse study (Chapter 2), the PGPR compound provided some control of *P. syringae* pv. *tomato*, though not to the same degree as ASM and copper. ASM, alone or in combination with the PGPR compound, controlled bacterial speck disease as well as or better than copper. All treatments reduced the amount of bacterial DNA detected in field samples, with very low amounts observed in plants treated with both activators. This detrimental effect on bacterial growth in plants treated with both plant activators coincides with a large increase in jasmonic acid signaling, indicating a possible role for this pathway in reducing pathogen populations. The PGPR compound inconsistently provided a boost in yield, either alone or in combination with ASM and no decrease in yield was observed in ASM-treated plants.

The salicylic acid and ethylene signaling pathways were not consistently activated in ASM-treated plants prior to pathogen inoculation, though this result is consistent with Chapter 3, as samples were taken seven days after ASM application. Under low disease pressure, ASM-treated plants failed to re-activate these signaling pathways above levels observed prior to inoculation with *P. syringae* pv. *tomato*. Additionally, ASM (which was applied 12 hours prior to inoculation) also did not induce re-activation of the pathways. In contrast, under heavy disease pressure and hail storm-induced wounding, both inoculated and noninoculated plots showed increasing salicylic acid and ethylene signaling pathway activation, though levels dropped dramatically at symptom development. A greater response was observed in inoculated plots and plants treated with ASM were induced to a higher level than ASM and PGPR-treated plants. This result indicates possible negative cross-talk

between signaling pathways, though significant effect on disease control was observed. No priming effects of the PGPR compound were detected in this system, though possible alternative marker genes could measure responses that correlated with the observed disease control.

Wound-induced signaling was minimal under low disease pressure and did not correlate with amount of disease (unlike results found in Chapter 2). The jasmonic acid pathway was activated following a hail storm in both inoculated and noninoculated plots, though induction was significantly lower than levels observed at subsequent time points in inoculated plants. Disease severity was reflected in wound-induced expression in untreated control, PGPR and ASM-treated plots. Copper and the combination of plant activators appeared to have compounded jasmonic acid signaling when plants were wounded and inoculated with the pathogen, though both provided disease control as good as ASM alone.

Implications for disease management programs. Collective results from these studies (Chapters 2-4) demonstrate that the PGPR compound provided some control of *P. syringae* pv. *tomato*, though it was not to commercially acceptable levels. Combined use of the activators provided disease control as good as copper or ASM alone, and in some cases had a positive impact on yield. Synthesis of these data indicate that PGPR may provide a yield boost under certain environmental conditions while ASM is a viable and consistent management tool for *P. syringae* pv. *tomato* in greenhouse and field production in New York. Additionally, use of both activators in conjunction provides no negative effect on disease control. Further studies on the combined use of plant activators are necessary to establish the optimal

conditions to reap the yield enhancing and disease control capabilities of these compounds.

Findings from these studies can be incorporated into previous tomato growing recommendations from planting to harvest (Reiners and Petzoldt 2007; Smart, Borsick, and Lange 2005). At planting, growers should sterilize work surfaces and propagation materials, using certified disease-free seed and pathogen resistant/tolerant varieties when available. Weed, insect and water management is important in both the greenhouse and field to help lower the dissemination of pathogens. When visibly infected plants are found in the greenhouse, their corresponding flat should be removed as well. Copper and ASM provide sufficient control of *P. syringae* pv. *tomato* in the greenhouse, whereas the PGPR compound is not recommended.

A minimum two year rotation away from susceptible crops should be practiced for bacterial disease management. The PGPR compound could provide some yield or disease control benefits under stressful conditions, though more research is necessary before use of this product is recommended. Under low to moderate disease pressure, copper and ASM are equally effective, though use of ASM is cost prohibitive. When conditions are conducive to bacterial speck development (cool, wet weather), incorporation of ASM into a management scheme is economically feasible, possibly reducing the number of copper sprays required.

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REFERENCES

Chester, K. S. 1933. The problem of acquired physiological immunity in plants. *Quarterly Review of Biology* 8 (2):129-154.

Chester, K. S. 1933. The problem of acquired physiological immunity in plants (continued). *Quarterly Review of Biology* 8 (3):275-324.

Kuc, J. 1982. Induced immunity to plant disease. *BioScience* 32 (11):854-860.

McSpadden Gardener, B.B., and Fravel, D.R. 2002. Biological control of plant pathogens: Research, commercialization, and application in the USA. *Plant Health Progress*.

Reiners, S., and Petzoldt, C., eds. 2007. *The integrated crop and pest management guidelines for vegetables*. Ithaca: Cornell University.

Smart, C.D., Borsick, M.A., and Lange, H.W. 2005. Producing disease-free transplants. Proceedings of the Empire State Fruit and Vegetable Expo, Syracuse.

APPENDIX ONE

*EFFECTS OF PLANT GROWTH-PROMOTING RHIZOBACTERIA ON BELL PEPPER PRODUCTION AND GREEN PEACH APHID INFESTATIONS IN NEW YORK

ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) are known in various cropping systems to increase plant growth and vigor, as well as induce resistance to pathogens and pests. A commercial soil amendment containing a mixture of two species of *Bacillus* PGPR (*B. subtilis* and *B. amyloliquefaciens*), was evaluated for impact on germination and initial growth of bell pepper plants, efficacy against the green peach aphid, *Myzus persicae* Sulzer, and yield enhancement. Studies in the greenhouse revealed that pepper germination rate and dry weight of seedlings grown with or without *Bacillus* spp. did not differ significantly. In the field, the PGPR did not significantly reduce aphid populations compared to control plants, whereas imidacloprid was highly effective. An increase in yield compared to control plants was observed in the 2003 season, but not the following two seasons. Aphid pressure was high in 2003, and plants grown in the presence of *Bacillus* spp. exhibited substantial tolerance to aphids. That is, there were significantly higher populations of the green peach aphid on both control and PGPR-treated plants compared to imidacloprid treated plants. However, fruit yield in the *Bacillus* spp. treatment was significantly greater than yield in the control

treatment and similar to yield in insecticide-treated plots. *Bacillus* PGPR could be useful in a *M. persicae* management program for pepper plants grown in locations with consistently high aphid pressure.

*Herman, M.A.B., Nault, B.A., and Smart, C.D. 2008. Effects of plant growth-promoting rhizobacteria on bell pepper production and green peach aphid infestations in New York. Crop Protection. (*In Press*).

INTRODUCTION

Rhizobacteria colonize plant roots and consume root exudates and lysates (Antoun and Prevost 2006; Pieterse et al. 2002). Certain strains are referred to as plant growth-promoting rhizobacteria (PGPR), which can be used as inoculant biofertilizers (Kennedy et al. 2004). These bacteria include species of *Pseudomonas* and *Bacillus*, both of which provide direct and indirect effects on plant growth and pest resistance (Kennedy, Choudhury, and Kecskes 2004; Nelson 2004; Persello-Cartieaux et al. 2003). PGPR can directly benefit plant growth by increasing nitrogen uptake, synthesis of phytohormones, solubilization of minerals, and iron chelation (Bowen and Rovira 1999). While a positive impact of PGPR on initial growth of bell pepper, *Capsicum annuum* L., has been previously described (Garcia et al. 2004; Joo et al. 2005; Kokalis-Burelle et al. 2002; Russo 2006), none of the previous studies were done under environmental and cultural conditions found in the Northeastern United States. Thus, the utility of PGPR as inoculant biofertilizers in this region are not well understood.

Another direct effect of some PGPR is suppression of soil-borne pathogens by production of siderophores, antimicrobial metabolites, or competing for nutrients and/or niches (Nelson 2004). Indirectly, some PGPR stimulate an increase in resistance to pathogens and pests that feed on leaves by activating the formation of physical and chemical barriers in the host (Persello-Cartieaux, Nussaume, and Robaglia 2003; Ryu et al. 2003). This phenomenon is referred to as induced systemic resistance, and activates the plant jasmonic acid and ethylene signaling pathways (Bostock 2005; Kloepper et al. 2004; Pieterse et al. 2002).

Induced resistance is a phenomenon documented in many plant-insect and plant-pathogen interactions (Conrath et al. 2006; Stout et al. 2006; Tuzun and Bent 2006; Zehnder et al. 1997; Zehnder et al. 2001). The concept of activating a plant's defense pathways to control pests in agriculture is appealing, though difficult to implement effectively. There are several examples of plants treated with PGPR, or with chemical inducers of the same plant signaling pathways, which show a decrease in insect herbivory. Zehnder et al. (1997) used PGPR to reduce feeding by the spotted cucumber beetle, *Diabrotica undecimpunctata howardi* Barber, six-to ten-fold on cucurbits. Boughton et al. (2006) reported that plants treated with defense elicitors caused green peach aphid, *Myzus persicae* Sulzer, populations to increase significantly more slowly than populations on control plants (Boughton et al. 2006). Additionally, white clover and *Medicago* plants grown in the presence of a *Pseudomonas*-like PGPR were better able to resist effects of blue-green aphids, *Acyrthosiphon kondoi* Shinji (Kempster et al. 2002). Stout et al. (2002) speculated that the delay in population growth and population size of cotton aphids, *Aphis gossypii* Glover, on cucumbers was due to a *Bacillus*-containing PGPR treatment (Stout et al. 2002). Several *Bacillus* PGPR species applied to tomato as seed treatments were found to reduce whitefly nymph densities 40-43%, but did not consistently decrease the severity of whitefly-transmitted tomato mottle virus or increase yield (Murphy et al. 2000).

The green peach aphid, *M. persicae*, is a pest of pepper in New York, attacking over 75% of the acreage annually (Frantz et al. 2004). Large numbers of aphids can reduce plant vigor and cause defoliation (Figure A.1). While many insecticides are registered for *M. persicae* control on pepper,

Figure A.1. Green peach aphids on a pepper leaf.

there is a need for biologically based products to control infestations. A PGPR would be of great value, especially to conserve natural enemies and to avoid potential problems encountered when some insecticides fail to control populations that have developed resistance (Devonshire 1989; Minks and Harrewijn 1989; Reiners and Petzoldt 2007; Wang et al. 2002).

The goal of this study was to determine the utility of a commercially available *Bacillus* PGPR product for improving plant growth and controlling *M. persicae* on field-grown peppers in New York. The hypotheses were that the *Bacillus* spp. would (1) enhance germination and initial plant growth of pepper seedlings before transplanting in the field, (2) reduce populations of *M. persicae* on pepper and (3) contribute to greater fruit yield.

MATERIALS AND METHODS

Seedling production and Bacillus spp. treatment. Pepper, c.v. **'**Camelot', seeds were sown in Cornell mix, a soilless peat mixture, with perlite and vermiculite (4:1:1) in 256 (2003) or 128 (2004 and 2005) cell plug trays (Griffin Greenhouse and Nursery Supplies, Auburn NY, USA) commonly used for pepper transplant production in New York, USA. Each tray was 42 cm x 25.5 cm, with a cell size of 1.5 cm x 1.5 cm or 2 cm x 2 cm for the 256 and 128 cell plug trays, respectively. Nitrogen, phosphorus and potassium (10-5-10) fertilizer was added at a rate of 2.67 kg/m^3. The PGPR-containing product BioYield ™ (Bayer CropScience LP, Research Triangle Park, NC, USA) was mixed with potting mix prior to planting (1.2 kg/m^3). The formulation contains two bacterial strains, *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a. Plants were grown in a greenhouse under natural sunlight with temperatures

of 23-26°C (day) and 20-22°C (night). In Geneva, NY, USA, the photoperiod is approximately 15 h L: 9 h D from mid-May to mid-June. One week prior to field planting, seedlings were moved to an outdoor coldframe. Plants were fertilized with liquid fertilizer (15:30:15 N-P-K) prior to field planting.

Bacillus spp. impact on germination and seedling size. In the greenhouse, the germination rate (number germinated seeds out of total seeds planted over time) was compared between plants grown in *Bacillus* spp.-treated and untreated potting mix in 2004 and 2005. In 2004, numbers of germinated seeds in each 128 cell plug tray was recorded twice per week for a month for a total of nine observations. In 2005, germination was recorded every 3-5 days for three weeks after sowing seed for a total of five observations.

Dry weight of 20 plants grown in either *Bacillus* spp.-treated or untreated potting mix was measured as previously described (Still and Pill 2004), with a slight modification. Shoots and roots of five-week-old plants were washed and dried separately, and tissue was dried in paper bags in a 65°C oven for five days.

Field experiments to evaluate performance of Bacillus spp. Field experiments were conducted at the New York State Agricultural Experiment Station's Fruit and Vegetable Research Farm in Geneva, NY, USA from 2003 to 2005. In all experiments, six-week-old transplants were hand-planted in the field on 17 June, 16 June and 8 June, respectively. Seedlings were transplanted into beds covered with black plastic mulch with plants spaced at 30.5 cm intervals within the row. Each plot consisted of two 6.1-meter long rows that were spaced 0.9 m apart with 20 plants per row. Peppers were fertilized, irrigated

and weeds controlled following typical production practices in western NY, USA (Reiners and Petzoldt 2007).

Manipulating aphid densities in pepper using esfenvalerate. The ability to generate high populations of aphids was important to enable evaluation of the impact of *Bacillus* spp. on *M. persicae*. The premise behind this approach was to utilize an insecticide to which *M. persicae* populations would be resistant, whereas populations of natural enemies would be eliminated. In the absence of natural enemies, *M. persicae* populations would increase. To insure the utility of this approach, field plots of peppers were either treated with a low rate of the broad-spectrum pyrethroid insecticide esfenvalerate (Asana XL, DuPont, Wilmington, DE, USA) or left untreated. Treatments were arranged in a randomized complete block design and replicated four times. Esfenvalerate was applied twice, on 30 July and 19 August, 2003 at a rate (4.79 ml/ha) below that which is recommended by the manufacturer. *M. persicae* populations were recorded weekly, five times during the months of August and September. Numbers of wingless aphids were assessed in the field, by eye and recorded from twenty randomly collected leaves from one row per plot. The plot row sampled was alternated each week to prevent unequal removal of foliage.

Impact of Bacillus spp. on aphid control. Aphid populations on pepper plants were compared among four treatments including: 1) plants grown in *Bacillus* spp.-treated potting mix (BioYield), 2) plants treated with the conventionally used systemic insecticide imidacloprid (Admire 2F, Bayer CropScience LP, Research Triangle Park, NC, USA), 3) plants grown in the presence of *Bacillus* spp. and treated with imidacloprid, and 4) untreated control plants.

Treatments were arranged in a randomized complete block design with four replications. Imidacloprid was applied as an in-furrow drench at planting at a rate of 19.15 ml/ha. Each year, the entire test site was treated with a low rate (4.79 ml/ha) of esfenvalerate to increase *M. persicae* populations to allow evaluation of treatments under high aphid pressure. In 2003, applications were made on 30 July and 19 August, and in 2004 applications were made on 16 July, and 6 and 9 August. In 2005, one application was made on 25 July.

In 2003 and 2004, numbers of wingless *M. persicae* was assessed periodically in August and September. Densities were recorded using the same method described above.

Impact of Bacillus spp. on fruit yield. All fruit greater than 5 cm in diameter were harvested, counted and weighed during each of the three seasons, from each treatment. In 2003, plots were harvested on 11 and 18 September, in 2004 plots were harvested on 20 August, 3 and 21 September, and in 2005 plots were harvested on 19 August and 8 September. Yield comparisons among treatments were made at each harvest date, as well as for the total season yield for each treatment.

Statistical analyses. All data were analyzed using SAS software version 9.1 (Cary, NC, USA). Numbers of germinated seedlings over time were analyzed for interactions between time and treatment using a univariate mixed-effects analysis of variance (Proc Mixed) and evaluated using least squares means. Percentage of germinated seed at each time point, root and shoot dry weights, aphid densities, and yield (fruit number and weight) were assessed using a one-way analysis of variance (Proc GLM), and means compared with a Fisher's Protected LSD at $P<0.05$. For analysis of aphid densities and season

aphid totals, this technique was preferred over a repeated measures analysis of variance because the study was not designed to compare population trends among treatments. To stabilize variance in aphid data sets prior to analysis, the transformation $\log_{10}(x + 1)$ was used. Untransformed means are presented.

RESULTS

Impact of Bacillus spp. on germination and seedling development. The *Bacillus* spp.-treated potting mix did not affect germination or enhance seedling development. In 2004 and 2005, the rate of germination was not significantly different between treated and untreated control plants (F= 1.17; df = 1, 221; *P*= 0.19, F= 0.19; df = 1, 33; P= 0.67, respectively). In 2004, average germination rates were 1.22 (untreated) and 1.26 (BioYield) seedlings per day, while in 2005 rates were 4.49 (untreated) and 4.52 (BioYield) seedlings per day. Additionally, there were no significant differences in numbers of germinated seed at any recording time in either year (F= 1.25; df = 1, 221; *P*= 0.27, F= 0.23; df = 1, 33; *P*= 0.64, respectively). In 2004, there was a very low percent germination in untreated and *Bacillus* spp.-treated potting mix (29.8% and 30.3%, respectively), while in 2005 a much higher percent germination was observed at 98.7% for untreated and 97.4% for *Bacillus* spp.-treated mix.

Five weeks after planting, pepper root and shoot dry weights did not differ between treatments (root weight: F= 1.58; df = 20; *P*= 0.16; shoot weight: F= 1.11; df = 20; *P*= 0.41). Peppers grown in the presence of *Bacillus* spp. had an average shoot dry weight of 0.87 g, while shoots of untreated

plants averaged 0.80 g. Root dry weight for *Bacillus*-treated peppers and untreated peppers averaged 0.41 g and 0.42 g, respectively.

Effect of esfenvalerate on aphid densities. The season total number of aphids in plots treated with esfenvalerate was five times greater than the total number in the untreated control (F= 15.25; df = 1, 3; *P*= 0.03) (Table A.1). A dramatic separation in aphid densities between treatments occurred on 3 and 9 September, 2 and 3 weeks after the second application of esfenvalerate was applied, respectively (Table A.1).

Efficacy of Bacillus-induced resistance to control green peach aphids. Peppers grown in the presence of *Bacillus* spp. had fewer aphids compared to the control, although not significantly fewer, in both 2003 and 2004 (Table A.2). In contrast, imidacloprid provided excellent control of *M. persicae* in both years (2003: F= 18.19; df = 5; *P*= 0.001; 2004: F= 19.53; df = 5; *P*= 0.0001) (Table A.2). In 2004, the combination of imidacloprid and *Bacillus* spp. did not provide a greater level of control than that provided by imidacloprid alone (Table A.2).

Infestations of *M. persicae* were much higher in 2003 than in 2004 (Table A.2). In 2003, the mean number of aphids per leaf during peak infestation in control plots was only 2.7 times greater than the mean number in *Bacillus* spp. treated plots, but 500 times greater than the mean number in imidacloprid treated plots. In 2004, the mean number of aphids per leaf during peak infestation in control plots was only 1.3 times greater than the mean number in *Bacillus* spp. treated plots, but 90 times greater than the mean number in imidacloprid treated plots. In 2005, the test site was not colonized by sufficient numbers of

Table A.1. Effect of esfenvalerate on wingless populations of *Myzus persicae* on bell pepper in NY in 2003.

Treatment	Mean number ± SE of wingless aphids per 20 leaves[a]					
	18 Aug	25 Aug	3 Sept	9 Sept	17 Sept	Season total[b]
Untreated	101 ± 29	106 ± 30	126 ± 61	97 ± 52	27 ± 13	457 a
Esfenvalerate[c]	44 ± 6	175 ± 30	900 ± 342	851 ± 175	48 ± 28	2022 b

[a]Data were transformed by $\log_{10}(x + 1)$ before analysis, but untransformed means are presented.

[b]Means within a column followed by different letters are significantly different (P>0.05, Fisher's LSD).

[c]Esfenvalerate treatments were applied on 30 Jul and 19 Aug. Few aphids were observed at the test site in late July, and therefore were not recorded.

Table A.2. Effect of *Bacillus* species and imidacloprid on wingless populations of *Myzus persicae* on bell pepper in NY in 2003 and 2004.

2003 Treatment[b]	Mean number ± SE of wingless aphids per 20 leaves[a]					
	18 Aug	25 Aug	3 Sept	9 Sept	17 Sept	Season total[c]
Control	44 ± 6	175 ± 30	900 ± 342	851 ± 175	48 ± 28	2022 a
Bacillus spp.	31 ± 8	122 ± 45	323 ± 143	567 ± 315	36 ± 24	1079 a
Imidacloprid	1 ± 1	1 ± 1	2 ± 1	2 ± 1	0 ± 0	6 b
2004 Treatment[b]	Mean number ± SE of wingless aphids per 20 leaves[a]					
	27 Aug	3 Sept	10 Sept	20 Sept		Season total[c]
Control	132 ± 56	135 ± 59	31 ± 11	5 ± 3		303 a
Bacillus spp.	57 ± 9	102 ± 43	17 ± 8	4 ± 1		179 a
Imidacloprid	2 ± 1	2 ± 1	1 ± 1	1 ± 0		5 b
Imidacloprid+ *Bacillus* spp.	0 ± 0	1 ± 0	0 ± 0	1 ± 0		2 b

[a]Data were transformed by $\log_{10}(x + 1)$ before analysis, but untransformed means are presented.

[b]Esfenvalerate was applied to all treatments, including the control on 30 July and 19 August 2003 and 16 July, 6 and 9 August 2004.

[c]Means within a column followed by the same letter are not significantly different (P>0.05, Fisher's LSD).

M. persicae to establish a pest infestation. The population was so low throughout the season that data were not collected.

Impact of Bacillus spp. on yield.

2003 Yield Study. The majority of fruit from the 2003 season was collected during the final harvest on 18 Sept. In the initial harvest (11 Sept.), mean number of fruit did not differ among treatments ($P>0.05$) (Table A.3). Yet, peppers grown in *Bacillus* spp.-treated potting mix weighed significantly more than control and imidacloprid-treated peppers ($F= 7.00$; $df= 5$; $P= 0.02$) (Table A.3). In the final harvest, yield was significantly different between all treatments; yield was highest in imidacloprid-treated peppers and lowest in the untreated control (number of fruit: $F= 21.37$; $df= 5$; $P= 0.001$; weight of fruit: $F= 12.01$; $df= 5$; $P= 0.004$) (Table A.3). The season total number and weight of fruit was greater in treated plots than the control, but did not differ significantly among imidacloprid and *Bacillus* spp. treatments (number of fruit: $F= 11.34$; $df= 5$; $P= 0.005$; weight of fruit: $F= 12.09$; $df= 5$; $P=0.004$) (Table A.3).

2004 Yield Study. Peppers were harvested three times throughout the 2004 season, with a very small initial harvest on 20 Aug. No difference between treatments was observed in fruit number or weight in the first two harvests, or for season totals (number of fruit: $F= 2.4$; $df= 6$; $P= 0.11$; weight of fruit: $F= 1.93$; $df= 6$; $P=0.18$) (Table A.4). In the third harvest, imidacloprid-treated plants had significantly less fruit than the untreated control, but did not differ significantly from the other treatments ($F= 3.69$; $df= 6$; $P= 0.04$).

Table A.3. Effect of *Bacillus* species and imidacloprid insecticide on number and weight of bell pepper fruit at NY, USA in 2003

Treatments[a]	1st Harvest 11 Sept		2nd Harvest 18 Sept		Total yield	
	Mean no. of fruit[bc]	Mean weight (kg/plot)	Mean no. of fruit	Mean weight (kg/plot)	Mean no. of fruit	Mean weight (kg/plot)
Control	20.8 a	3.3 b	73.3 c	9.2 c	94.0 b	12.5 b
Bacillus spp.	32.5 a	6.6 a	98.3 b	11.8 b	130.8 a	18.4 a
Imidacloprid	29.5 a	4.6 b	127.3 a	15.5 a	156.8 a	20.0 a

[a]Esfenvalerate was applied to all treatments, including the control on 30 July and 19 August to increase the population of *M. persicae*.

[b]Means calculated based on 40 plants per plot.

[c]Means within a column sharing the same letters are not significantly different (P>0.05, Fisher's LSD).

Table A.4. Effect of *Bacillus* species, imidacloprid and both on total bell pepper yield at NY, USA in 2004 and 2005.

	1st Harvest 20 Aug		2nd Harvest 3 Sept		3rd Harvest 21 Sept		Total yield	
2004 treatment[a]	Mean no.[bc]	Mean wt[d]	Mean no.	Mean wt	Mean no.	Mean wt	Mean no.	Mean wt
Control	5.0 a	0.8 a	80.8 a	10.7 a	96.5 a	12.1 a	182.3 a	23.5 a
Bacillus spp.	5.3 a	0.9 a	56.8 a	7.1 a	66.8 ab	9.1 a	128.8 a	17.1 a
Imidacloprid	3.5 a	0.6 a	55.5 a	7.5 a	64.5 b	9.1 a	123.5 a	17.3 a
Imidacloprid +*Bacillus* spp.	4.8 a	0.8 a	56.5 a	7.3 a	76.3 ab	10.4 a	137.5 a	18.5 a

2005	1st Harvest 19 Aug		2nd Harvest 8 Sept		Total yield	
Treatment[a]	Mean no.[bc]	Mean wt	Mean no.	Mean wt	Mean no.	Mean wt
Control	60.3 a	6.7 a	43.3 a	5.0 a	103.5 a	11.7 a
Bacillus spp.	60.5 a	7.1 a	46.0 a	5.4 a	106.5 a	12.5 a
Imidacloprid	75.0 a	9.1 a	19.8 a	2.5 a	94.8 a	11.6 a
Imidacloprid +*Bacillus* spp.	85.3 a	11.0 a	16.0 a	2.0 a	101.3 a	13.0 a

[a]Esfenvalerate was applied to all treatments including the control on 16 July, 6 and 9 August 2004 and 25 July 2005 to increase the population of *M. persicae*.

[b]Mean number of fruit calculated based on 40 plants per plot.

[c]Means within a column sharing the same letters are not significantly different (P>0.05, Fisher's LSD).

[d]Mean fruit weight (kg) per 40 plant plot.

2005 Yield Study. The majority of peppers were harvested during the first of two harvests on 19 Aug. No difference between treatments was observed in fruit number or weight in either harvest (P>0.05) (Table A.4). Total number and weight of fruit were not different among the treatments (number of fruit: F= 1.45; df= 6; P= 0.29; weight of fruit: F= 1.97; df= 6; P=0.17) (Table A.4).

DISCUSSION

Several studies have reported the utility of *Bacillus* PGPR species for growth promotion and biological control of diseases of pepper (Garcia et al. 2004; Joo et al. 2005; Kokalis-Burelle et al. 2002; Russo 2006), but our study is the first to examine the efficacy against *M. persicae* in addition to impact on growth promotion and yield. Previous research on pepper transplants grown in Florida found the mixture of *B. subtilis* GB03 and *B*. *amyloliquefaciens* IN937a to increase transplant vigor (Kokalis-Burelle et al. 2002). The same mixture was used in the current study and found to have no impact on the rate of seed germination, or on early seedling growth. A difference in pepper varieties used in the two studies could explain this difference. Alternatively, the variation in growing conditions between the warmer Florida climate and that of the cooler climate in New York may also play a role in the increased vigor seen in the previous study compared to the present study. Two other previous reports of increased pepper plant growth following inoculation with PGPR utilized *B. cereus* MJ-1 (Joo et al. 2005) and *B. licheniformis* (Garcia et al. 2004). In these studies, the difference in *Bacillus* spp. used could explain differences in results.

Our study followed germination rates in two seasons (2004 and 2005). The overall germination rate in 2004 was very low at approximately 30%. This

low rate was due to problems with greenhouse heating combined with cool spring temperatures. Even though the overall rate was low, *Bacillus* spp.-treated and untreated planting mix had statistically similar germination rates. In 2005, germination rates were much higher (approximately 98%), and again there were no significant differences between treatments. The germination rates between treatments were similar in each of the two seasons, with no difference observed between *Bacillus* spp.-treated and untreated planting mix. Therefore, we are confident that the low germination rate in 2004 did not have a greater impact on seed sown in treated versus untreated mix.

The ability to generate high populations of aphids was important for evaluating the impact of *Bacillus* spp. on *M. persicae*. Esfenvalerate is a broad-spectrum insecticide that at low rates is harsh on natural enemies, but does not harm *M. persicae* (Mowry 2005). Esfenvalerate was found to be highly effective in 2003, with *M. persicae* populations increasing to significantly higher levels in treated plots. A delay in the population increase was observed, possibly due to a time delay between killing natural enemies (predators and parasitoids) of aphids, and *M. persicae* reproduction. The final aphid collection of 2003 showed no difference in aphid levels between esfenvalerate and untreated plants. This was due to naturally declining aphid populations at season end. The rapid decline in aphid populations is frequently due to a decrease in aphid fecundity associated with declining plant quality or weather induced mortality (Frazer 1988). Applications of esfenvalerate were effective in causing populations of *M. persicae* to increase on pepper in 2003 and 2004, but not in 2005.

M. persicae populations were high in 2003, moderate in 2004 and did not develop on peppers in 2005. Environmental factors such as high levels of precipitation early in the season followed by excessive heat and radiation later in the season could be responsible, as these factors impact aphid population dynamics (Carver 1988). In June 2005, there was 1224.28 cm of precipitation (a 65% increase from 2004, 37% increase from 2003). Additionally, the average high temperature for June, July and August was 27.9 °C, with 35 days over 30 °C. There were only 8 and 5 days with high temperatures above 30°C in 2003 and 2004, respectively.

Growing plants in the presence of *Bacillus* PGPR did not control aphid infestations when *M. persicae* populations were present in 2003 and 2004. In contrast, imidacloprid was very successful in aphid control in both years. While the differences were not significant, there were fewer aphids colonizing plants grown in the presence of *Bacillus* PGPR compared with untreated control plants at each rating date in both 2003 and 2004. While the PGPR clearly do not control aphids to the level of imidacloprid, there is certainly a trend toward fewer aphids on treated plants. Future studies may enable us to better understand these interactions.

Differences in yield between plants grown with PGPR, imidacloprid treated and control pepper plants were observed in 2003, with plants grown in the presence of *Bacillus* spp. producing fruit with a significantly greater weight in the first harvest on 11 Sept. This increase in fruit weight would be important in commercial production as a premium is paid for early fruit. By the second harvest on 18 Sept, the impact of huge aphid populations reduced the yield on both *Bacillus* spp-treated and control plants compared to imidacloprid-treated

plants. Interestingly, at this second harvest the *Bacillus* spp.-treated plants still had a significantly higher yield in both number and weight of fruit compared to the control plants. This increase in yield was not observed in plants grown in the presence of *Bacillus* spp. during the 2004 and 2005 seasons. This could be because the yield and growth benefits of this PGPR may only be seen under stressful conditions, such as high aphid pressure. Previous studies have shown PGPR to aid in plant responses to salt stress, drought and phosphorus deficiency (Mayak et al. 2004; Saravanakumar and Samiyappan 2007; Wittenmyer and Merbach 2005)

We hypothesized that the *Bacillus* PGPR compound would improve plant growth, control *M. persicae* infestations, and increase pepper yield in New York, USA. Results indicated that the *Bacillus* PGPR had no statistically significant effect on germination, seedling growth or control of *M. persicae*. Under high aphid pressure, as observed in 2003, plants grown in the presence of *Bacillus* PGPR tolerated economically damaging levels of *M. persicae* without a reduction in yield. *Bacillus* PGPR could be useful in a *M. persicae* management program for pepper plants grown in locations with consistently high aphid pressure, though subsequent research in years of high aphid pressure would be necessary to fully support this conclusion. In a pepper management scheme, utilization of a broad-spectrum product to control European corn borer, *Ostrinia nubilalis* Hübner, infestations could cause *M. persicae* populations to increase rapidly. However, control of aphids may not be necessary if a *Bacillus* PGPR is used. Moreover, the combination of *Bacillus* PGPR and conservation of naturally occurring biological control

organisms in pepper fields may further suppress aphid populations and preclude the need for aphid control using insecticides.

REFERENCES

Antoun, H., and Prevost, D. 2006. Ecology of Plant Growth Promoting Rhizobacteria. In *PGPR: Biocontrol and Biofertilization*, edited by Z. A. Siddiqui. Dordrecht: Springer.

Bostock, R. M. 2005. Signal crosstalk and induced resistance: straddling the line between cost and benefit. *Annual Review of Phytopathology* 43:545-580.

Boughton, A. J., Hoover, K., and Felton, G. W. 2006. Impact of chemical elicitor applications on greenhouse tomato plants and population growth of the green peach aphid, *Myzus persicae*. *Entomologia Experimentalis Et Applicata* 120 (3):175-188.

Bowen, G. D., and Rovira, A. D. 1999. The rhizosphere and its management to improve plant growth. *Advances in Agronomy* 66:1-102.

Carver, M. 1988. Biological control of aphids. In *Aphids: their biology, natural enemies, and control, Volume 2C*, edited by A. K. Minks and P. Harrewijn. Amsterdam: Elsevier Science Publishers.

Conrath, U., Beckers, G. J. M., Flors, V., Garcia-Agustin, P., Jakab, G., Mauch, F., Newman, M. A., Pieterse, C. M. J., Poinssot, B., Pozo, M. J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D., Zimmerli, L., Mauch-Mani, B., and Grp, Prime-A-Plant. 2006. Priming: getting ready for battle. *Molecular Plant-Microbe Interactions* 19 (10):1062-1071.

Devonshire, A.L. 1989. Resistance of aphids to insecticides. In *Aphids: their biology, natural enemies, and control, Volume 2C*, edited by A. K. Minks and P. Harrewijn. Amsterdam: Elsevier Science Publishers.

Frantz, J. A., Gardner, J., Hoffmann, M. P., and Jahn, M. M. 2004. Greenhouse screening of *Capsicum* accessions for resistance to green peach aphids (*Myzus persciae*). *Hortscience* 39 (6):1332-1335.

Frazer, B.D. 1988. Predators. In *Aphids: their biology, natural enemies and control, Volume 2B*, edited by A. K. Minks and P. Harrewijn. Amsterdam: Elsevier Science Publishers.

Garcia, J. A. L., Probanza, A., Ramos, B., Palomino, M. R., and Manero, F. J. G. 2004. Effect of inoculation of *Bacillus licheniformis* on tomato and pepper. *Agronomie* 24 (4):169-176.

Joo, G. J., Kim, Y. M., Kim, J. T., Rhee, I. K., Kim, J. H., and Lee, I. J. 2005. Gibberellins-producing rhizobacteria increase endogenous gibberellins content and promote growth of red peppers. *Journal of Microbiology* 43 (6):510-515.

Kempster, V. N., Scott, E. S., and Davies, K. A. 2002. Evidence for systemic, cross-resistance in white clover (*Trifolium repens*) and annual medic (*Medicago truncatula* var *truncatula*) induced by biological and chemical agents. *Biocontrol Science and Technology* 12 (5):615-623.

Kennedy, I. R., Choudhury, A. T. M. A., and Kecskes, M. L. 2004. Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? *Soil Biology & Biochemistry* 36 (8):1229-1244.

Kloepper, J. W., Ryu, C. M., and Zhang, S. A. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94 (11):1259-1266.

Kokalis-Burelle, N., Vavrina, C. S., Rosskopf, E. N., and Shelby, R. A. 2002. Field evaluation of plant growth-promoting Rhizobacteria amended transplant mixes and soil solarization for tomato and pepper production in Florida. *Plant and Soil* 238 (2):257-266.

Mayak, S., Tirosh, T., and Glick, B. R. 2004. Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant Physiology and Biochemistry* 42 (6):565-572.

Minks, A.K., and Harrewijn, P., eds. 1989. *Aphids: their biology, natural enemies, and control, volume C*. Amsterdam: Elsevier Science Publishers.

Mowry, T. M. 2005. Insecticidal reduction of Potato leafroll virus transmission by *Myzus persicae*. *Annals of Applied Biology* 146 (1):81-88.

Murphy, J. F., Zehnder, G. W., Schuster, D. J., Sikora, E. J., Polston, J. E., and Kloepper, J. W. 2000. Plant growth-promoting rhizobacterial mediated protection in tomato against *Tomato mottle virus*. *Plant Disease* 84 (7):779-784.

Nelson, L.M. 2004. Plant growth promoting rhizobacteria (PGPR): prospects for new inoculants. In *Plant Management Network*.

Persello-Cartieaux, F., Nussaume, L., and Robaglia, C. 2003. Tales from the underground: molecular plant-rhizobacteria interactions. *Plant Cell and Environment* 26 (2):189-199.

Pieterse, C. M. J., Van Wees, S. C. M., Ton, J., Van Pelt, J. A., and Van Loon, L. C. 2002. Signalling in rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. *Plant Biology* 4 (5):535-544.

Reiners, S., and Petzoldt, C., eds. 2007. *The integrated crop and pest management guidelines for vegetables*. Ithaca: Cornell University.

Russo, V. M. 2006. Biological amendment, fertilizer rate, and irrigation frequency for organic bell pepper transplant production. *Hortscience* 41 (6):1402-1407.

Ryu, C. M., Hu, C. H., Reddy, M. S., and Kloepper, J. W. 2003. Different signaling pathways of induced resistance by rhizobacteria in *Arabidopsis thaliana* against two pathovars of *Pseudomonas syringae*. *New Phytologist* 160 (2):413-420.

Saravanakumar, D., and Samiyappan, R. 2007. ACC deaminase from Pseudomonas fluorescens mediated saline resistance in groundnut (Arachis hypogea) plants. *J Appl Microbiol* 102 (5):1283-92.

Still, J.R., and Pill, W.G. 2004. Growth and stress tolerance of tomato seedlings (*Lycopersicon esculentum* Mill.) in response to seed treatment with paclobutrazol. *Journal of Horticultural Science & Biotechnology* 79 (2):197-203.

Stout, M. J., Zehnder, G. W., and Baur, M. E. 2002. Potential for the use of elicitors of plant resistance in arthropod management programs. *Archives of Insect Biochemistry and Physiology* 51 (4):222-235.

Stout, M. J., Thaler, J. S., and Thomma, B. P. H. J. 2006. Plant-mediated interactions between pathogenic microorganisms and herbivorous arthropods. *Annual Review of Entomology* 51:663-689.

Tuzun, S., and Bent, E., eds. 2006. *Multigenic and induced systemic resistance in plants*. New York: Springer Science+Business Media, Inc.

Wang, K. Y., Liu, T. X., Yu, C. H., Jiang, X. Y., and Yi, M. Q. 2002. Resistance of *Aphis gossypii* (Homoptera : Aphididae) to fenvalerate and imidacloprid and activities of detoxification enzymes on cotton and cucumber. *Journal of Economic Entomology* 95 (2):407-413.

Wittenmyer, L., and Merbach, W. 2005. Plant responses to drought and phosphorus deficiency: contribution of phytohormones in root-related processes. *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* 168 (4):531-540.

Zehnder, G., Kloepper, J., Tuzun, S., Yao, C. B., Wei, G., Chambliss, O., and Shelby, R. 1997. Insect feeding on cucumber mediated by rhizobacteria-induced plant resistance. *Entomologia Experimentalis Et Applicata* 83 (1):81-85.

Zehnder, G. W., Murphy, J. F., Sikora, E. J., and Kloepper, J. W. 2001. Application of rhizobacteria for induced resistance. *European Journal of Plant Pathology* 107 (1):39-50.