OPTIMIZING THE EFFICACY AND ENVIRONMENTAL FITNESS OF A COMMERCIAL *PSEUDOMONAS* BACTERIAL BIOCONTROL PRODUCT FOR THE CONTROL OF TURFGRASS DISEASE.

By

Liewei Yan

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTERS OF SCIENCE

Plant Pathology

2011

ABSTRACT

OPTIMIZING THE EFFICACY AND ENVIRONMENTAL FITNESS OF A COMMERCIAL *PSEUDOMONAS* BACTERIAL BIOCONTROL PRODUCT FOR THE CONTROL OF TURFGRASS DISEASE.

By

Liewei Yan

A fungicide/antibiotic-producing bacterial strain of *Pseudomonas aureofaciens* Tx-1 (Tx-1) has been commercialized for golf course use with some success in recent years. To achieve adequate control it must be applied daily. This may be partially explained by UV susceptibility of Tx-1, which is a common limitation of biocontrol agents. Results from various wavelengths of ultraviolet radiation (A, B, C) exposure tests showed a significant drop in Tx-1 survivors following increased dosage. Significant improvement of UV tolerance was achieved and demonstrated in all UV exposure tests by mixing a sunscreen. In an attempt to identify the most UV-resistant individual cells, Tx-1 was exposed to intensive UV light (UV-C) for various lengths of time (*in vitro*). Cells (10TC30) with significantly improved survival rate after 7 J/m^2 UV-C irradiation was found followed by 10 cycles of 42 J/m^2 of UVC exposure. Field data of dollar spot and anthracnose control studies showed that applied at high concentration ($10^7 CFU/cm^2$), Tx-1 sunscreen mixture and the strain 10TC30 provided significant disease control compared with non-treated control in the field study. Population sizes of the treatments were monitored, and results demonstrate the improved percentage of survival of Tx-1 with the protection of sunscreen.

To all the lovely people in Vargas's Lab, Paul, Nancy, Ron, and Joe

ACKNOWLEDGMENTS

I offer my sincerest gratitude and thanks to Dr. Joe Vargas, who has supported me throughout my degree with his patience and knowledge. I attribute my degree to his effort and encouragement and without him this thesis could not have been completed. One simply would not wish for a better professor; To Dr. George Sundin for his guidance on my committee, especially in the field of microbiology and UV radiation impact on microbes, this comprised a great deal of my project; To Dr. Kevin Frank for his advice and insight for my research.

During my two years of graduate study, I have been blessed with a friendly and cheerful group of fellow lab members. I would like to thank Nancy Dykema and Ron Detweiler for their support and indoctrination throughout my project. Special thanks to Paul Giordano for the wonderful friendship and memory we shared for these two years.

Lastly, I would like to thank my patents for their love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Dollar spot, caused by *Rutstroemia floccosum syn. Sclerotinia homoeocarpa* F. T. Bennet, and anthracnose, caused by *Colletotrichum cereale* Manns sensulato Crouch, Clarke & Hillman, are two of the most common, destructive and costly fungal diseases of high maintenance turfgrasses (Vargas, 2005). Although successful management of these two diseases has been achieved throughout the years by the traditional usage of chemical fungicides, the development of pathogen resistance to multiple classes of systemic fungicides (Vargas, 2005; Murphy, 2008; Putman, 2010), the public's concern over chemical use on golf courses, and the increased number of EPA restrictions on chemical fungicide development and application, have prompted research on alternative disease control strategies.

Remarkable progress has been made at Michigan State University toward development of an effective biological control agent, the bacterium *Pseudomonas aureofaciens* strain Tx-1 (Tx-1), a soilborne bacterium capable of producing multiple antifungal compounds (Powell, 2000). *In vitro* studies have shown promising growth inhibition of both *R. floccosum* and *C. cereale* (Powell, 2000); Tx-1 was labeled by the EPA as a bio pesticide in 2000. A commercialized fermentation and delivery system was developed for Tx-1 (Dwyer, 1999). The system utilizes the irrigation system on golf courses for the distribution of Tx-1. This successful introduction of Tx-1 to golf courses has provided an alternative management for turfgrass diseases.

Due to the frequent mowing of turfgrass on golf courses, foliage is the primary infection site for many fungal diseases, including *R. floccosum* and *C. cereale*. Practical experience in the field has raised questions about the persistence of Tx-1. To further understand the persistence of Tx-1 on the foliage, Dwyer (1999) tested Tx-1 under field condition and showed that satisfactory

disease suppression of Tx-1 largely depends on high inoculum concentration and high application frequency. Lower concentration and application frequency of Tx-1 can result in significantly reduced disease control. This finding was further supported by his thesis work showing that field population of Tx-1 decline dramatically one month after the last application in the fall. Under high disease pressure during summer, Hardebeck (2004) found that diluted inoculum applied through the irrigation system failed to achieve sufficient dollar spot inhibition on fairways. In order to optimize the performance of Tx-1 in the field, it is necessary to improve the persistence of Tx-1 on turfgrass foliage.

Inhibition by ultra-violet radiation (UVR) is a common limitation of biological control use in the field (Harper, 2006). It has been reported that photo-degradation caused by two different wavelengths of UV within sun light , UV-A (320-400 nm) and UV-B (280-320 nm), inactivates most microbial insecticides and fungicides in field conditions (Tamez-Guerra et al., 2005; Hadapad, 2009). Based on preliminary research in the laboratory, the lack of persistence of *P. aureofacious* Tx-1 on turf foliage may be partially explained by its inhibition by UVR (Powell, pers. comm.). No related data have been published regarding solar UVR sensitivity of Tx-1. Numerous studies have been done in attempt to screen effective UVR sunscreen to improve residual tolerance to UV after application due to the lethal impacts of UVR on microbial biocontrol agents in the field, (Tamez-Guerra et al., 2005). Several promising UVR sunscreen for biological pesticides have been identified. A humic substances components, humic acid (HA), ubiquitously present in the environment (Trump, 2006), was previously found to be able to protect microbial organisms from UV radiation (Corin, 1998; Templeton, 2006; Cantwell, 2008; Lee, 2009), has low toxicity to microbial organism (Corin, 1998; Lee, 2009), and has been

tested on turfgrass before (Cooper, 1998; Dyke, 2009), could be a potential UVR sunscreen for Tx-1.

Therefore, we hypothesized that Tx-1 might be sensitive to solar UVR; by promoting the UVR tolerance of Tx-1, it is possible to improve its disease control efficacy in the field. The objective of this study was to investigate potential methods to improve UV tolerance of Tx-1. To achieve this goal, UV sensitivity of Tx-1 was first confirmed by challenging it with UV-A and UV-B. In an attempt to improve UV tolerance of Tx-1, three different approaches were taken: the first approach was to test the efficacy of HA as a UV protectant for Tx-1. The second approach was promoting pigmentation of Tx-1 by prolonging the incubation time. The third approach was to select a spontaneously UV-resistant Tx-1 cells by using intensive UV radiation (UV-C). In vitro bioassays were performed on isolates of TX-1 form the different treatments for their biocontrol efficacy after UV exposure. The efficacy tests were performed both *in vitro* an in the field.

LITERATURE CITED

Cantwell, R., Hofmann, R. and Templeton, M. (2008). Interactions between humic matter and bacteria when disinfecting water with UV light. Journal of Applied Microbiology **105,** 25-35.

Cooper, R.J., Liu, C., Fisher, D.S. (1998). Influence of Humic Substances on Rooting and Nutrient Content of Creeping Bentgrass. Crop Science **38,** 1639.

Corin, N., Backlund, P., Wiklund, T. (1998). BACTERIAL GROWTH IN HUMIC WATERS EXPOSED TO UV-RADIATION AND SIMULATED SUNLIGHT. Chemosphere**,** 1947-1958.

Dwyer, P.J., Jr., . (1999). Field Efficacy, Persistence, and Antibiotic Production of *Pseudomonas auerofaciens.* In Department of Botany and Plant Pathology (East Lansing: Michigan State University).

Dyke, A.V., Johnson, P.G., Grossl, P.R. (2009). Influence of humic acid on water retention and nutrient acquisition in simulated golf putting greens. Soil Use and Management **25,** 255-261.

Hadapad, A.B., Hire, R.S., Vijayalakshmi, N., Dongre, T.K. (2009). UV protectants for the biopesticide based on Bacillus sphaericus Neide and their role in protecting the binary toxins from UV radiation. Journal of Invertebrate Pathology **100,** 147-152.

Hardebeck, G.A., R. F. Turco, R. Latin, and Z. J. Reicher. (2004). Application of Pseudomonas aureofaciens Tx-1 through irrigation for control of dollar spot and brown patch on fairway height turf. HortScience **39(7),** 1750-1753.

Harper, D.R. (2006). Biological contorl by microoorganisms. In Encyclopedia of Life Sciences (John Wiley & Sons.

Lee, E., Lee, H., Jung, W., Park, S., Yang, D., Lee, K. (2009). Influences of humic acids and photoreactivation on the disinfection of Escherichia coli by a high-power pulsed UV irradiation. Korean Journal of Chemical Engineering **26,** 1301-1307.

Murphy, J., F. Wong, L. Tredway, et al. (2008). Best management practices for anthracnose on annual bluegrass turf. Golf Course Management **76(8),** 93-104.

Powell, J.F., Vargas, J. M., Jr., et al. (2000). Management of dollar spot on creeping bentgrass with metabolites of Pseudomonas aureofaciens (TX-1). Plant Disease **84,** 19-24.

Putman, A.I., Jung, G., and Kaminski, J. E. (2010). Geographic distribution of fungicideinsensitive Sclerotinia homoeocarpa isolates from golf courses in the northeastern United States. Plant Disease **94,** 186-195.

Tamez-Guerra, P., Rodriguez-Padilla, C., and Galán-Wong Luis, J. (2005). Solar Radiation (UV) Protectants for Microbial Insecticides. In Semiochemicals in Pest and Weed Control (American Chemical Society), pp. 127-147.

Templeton, M.R., Hofmann, R., Andrews, R.C. (2006). UV inactivation of humic-coated bacteriohages MS2 and T4 in water. Environmental Engineering Science **5,** 537-543.

Trump, J.I.V., Sun, Y., Coates, J.D. (2006). Microbial Interactions with Humic Substances. Advances in Applied Microbiology **60,** 55-96.

Vargas, J.M.J. (2005). Management of turfgrass diseases. . (Hoboken, New Jersey: John Wiley & Sons, Inc.).

LITERATURE REVIEW

Dollar spot

Dollar spot disease, caused by *Rutstroemia floccosum* syn. *Sclerotinia homoeocarpa* F. T. Bennet, affects almost all species of turfgrass on golf courses (Vargas, 2005). In order to meet the industrial quality and playability of the turfgrass, management of this disease is so expensive that it became the most economically important turfgrass disease in North America (Smiley, 2005).

Symptoms and Epidemiology

On low mowed turf, the disease can be seen as silver-dollar-sized spots with bleached-out color. Spots may coalesce into irregular shaped patches as the disease progresses and destroys large areas of turf. Bleached lesions with brown or reddish bands can be seen on longer blades. The brown bands do not develop on annual bluegrass [*Poa annua* L. f. *reptans* (Hausskn.) T. Koyama] (Vargas, 2005). Under high humidity conditions during early morning, the active growth of the pathogen is present as white fluffy mycelium on the foliage (Vargas, 2005). Dollar spot occurs on turf from late spring to the end of fall, but is typically most active from July through early September (Smiley, 2005). Foliar wetness and guttation fluid are crucial elements for disease development. Humid days at temperatures of 15 to 32 °C followed by cool nights are the conditions that favor the formation of guttation water and also the infection by *R. floccosum*. When suffering from low fertility and water stress, turfgrasses are more prone to dollar spot infection. The pathogen can be spread by mowers through infected turf clippings, and by irrigation water and foot traffic (Vargas, 2005).

Control and Management

Management strategies for dollar spot include cultural, chemical, and biological control methods. Cultural practices reduce the impact of dollar spot toward turfgrass and decrease the need for chemical fungicides. Maintaining adequate fertility during the disease season, especially via light and frequent applications of nitrogen, decreases the severity of dollar spot and increases the efficacy of chemical programs (Vargas, 2005). Keeping soil moisture at field capacity level of also facilitates disease control. Removing the dew during the morning is a common cultural method that has been adopted by golf course superintendents to prevent dollar spot (Vargas, 2005). In addition, Giordano (2010) and Nikolai (2001) have shown that rolling on golf course greens can inhibit dollar spot development.

Chemical control

Chemical fungicides are necessary in many cases to achieve acceptable disease control (Vargas, 2005). Both contact (multisite) and systemic (site-specific) fungicides can be used to manage dollar spot. The efficacy of contact fungicides, such as chlorothalonil, for long term disease control is limited by their short persistence on the turf. Their multisite mode of action largely reduces the risk of fungal resistance. Resistance to contact fungicides among *R. floccosum* has not been observed so far. Contact fungicides will be more efficient if applied as a preventive treatment. Based on chemical structures or biochemical modes of action, systemic fungicides commonly used on turfgrass can be categorized into four groups: benzimidazoles, dicarboximides, demethylation inhibitors (DMI), and QoI fungicides. Compared to contact fungicides, systemic fungicides are more effective for long term dollar spot suppression. However, the site-specific mode of action encourages the selection of *R. floccosum* strains with reduced sensitivity or even resistance to fungicides (Vargas, 2005). Repeated application of systemic fungicides has been shown to promote the populations of resistant strains and

compromise the efficacy of existing chemical treatments for managing dollar spot (Putman, 2010).

Anthracnose

Anthracnose, caused by *Colletotrichum cereale* Manns sensulato Crouch, Clarke & Hillman, is one of the most important and destructive diseases on golf course greens and fairways (Vargas, 2005). During warm weather when the pathogen is most active, *C. cereale* can cause both foliar anthracnose (foliar blight) and crown rot anthracnose (basal rot) (Smiley, 2005; Vargas, 2005). Unlike foliar anthracnose, crown rot anthracnose mainly destroys the crowns, the meristematic tissue of the annual bluegrass plants and kills the host (Smiley, 2005).

Symptoms and Epidemiology

On infected annual bluegrass putting greens or fairways, symptoms initially appear as yellow to bronze colored spots, from 0.25 to 0.50 inch (0.64-1.30 centimeters) in size (Vargas, 2005; Murphy, 2008). These spots may coalesce and form large, irregularly shaped patches as the disease progresses (Murphy, 2008). Infection on an individual leaf may first appear as reddish brown spots which may eventually spread to the entire blade (Vargas, 2005). On infected blades and stems, unique black reproductive structures (*acervuli*) with black spines (setae) that been produced by *C. cereale* can serve as a diagnostic tool for *C. cereale* (Vargas, 2005; Murphy, 2008). In the case of crown rot anthracnose, *acervuli* can be seen in darkened crowns (Vargas, 2005). Anthracnose is a major disease on annual bluegrass worldwide. It is most destructive during heat stress periods (Murphy, 2008). Foliar anthracnose normally occurs under high temperatures during summer and fall (Murphy, 2008); (Vargas, 2005). Crown rot anthracnose, on the other hand, occurs in both hot and cool seasons (Vargas, 2005; Murphy, 2008).

Cultural Management

Cultural management to improve host health during stress periods includes maintaining nitrogen fertility level around 0.5 lb. N /1000ft^2 per month, increasing the mowing height, irrigating lightly and frequently during the day and providing sufficient drainage to avoid soil saturation. These practices will not only facilitate the control of this disease and minimize the damage on greens and fairways, but also increase the effectiveness of chemical fungicide treatments (Vargas, 2005; Murphy, 2008). In addition to traditional cultural approaches to control anthracnose, recent studies demonstrate the potential usefulness of lightweight rolling and light frequent topdressing (Murphy, 2008).

Chemical control

Due to the epidemiology and destructiveness of anthracnose, preventive fungicide treatments control the disease more effectively than curative applications (Murphy, 2008). Among eight classes of fungicides that are available for anthracnose management, only the benzimidazole, DMI and QoI classes achieve satisfactory curative control (Murphy, 2008). The other classes of fungicides are more effective when applied preventively (Crouch, 2003; Towers, 2003). Similar to *R. floccosum,* C. *cereale* has became resistant to systemic fungicides for many years, which includes QoIs, benzimidazoles and DMI classes (Wong, 2004).

Biological control

Biological control has been used as a successful alternative strategy for managing soilborne or foliar diseases in a diverse range of crop systems (Baker, 1987; Cook, 1993). Aside from the efficacy of a suitable biological control organism, the strategies for maintaining population levels

and viability of the biological control agent are also needed to achieve satisfaction disease inhibition (Stack, 1988).

Pseudomonas aureofaciens was first discovered by Kluver (1956) from clay soil. *Pseudomonas* is a gram-negative bacterium, aerobic, abundant in agricultural rhizosphere, and belongs to the category of fluorescent *Pseudomonas* (Weller, 2007). The word "aureofaciens" literally means "made golden", which refers to its ability to produce orange pigments and turn the growth medium golden (Palleroni, 1984). The golden pigmented antibiotics have been categorized as phenazine antibiotics (Palleroni, 1984), which are N-containing heterocyclic molecules with a range of fungal suppression properties (Turner, 1986). Produced through a secondary metabolite pathway called the shikimic acid pathway (Turner, 1986), these antibiotic metabolites contribute 90% in fungal inhibition (Pierson, 1992). Studies on *P. aureofaciens* strain 30-84 revealed three phenazine components: phenazine 1-carboxylic acid (PCA), 2-hydroxy-phenazine-1-carboxylic acid (2-OH-PCA), and 2-hydroxy-phenazine (2-OH-PZ) (Pierson, 1992). Several hypotheses have been proposed regarding the biochemical mechanisms by which phenazine antibiotics suppress fungal growth. They include the generation of toxic oxygen species in intracellular space, inhibition of DNA replication, and disruption of the electron transport and energy cycling pathway (Pierson III, 1996). By using a phenazine-deficient mutant (Phz^-) of *P. aureofaciens* strain 30-84, Thomashow et al. (1990) demonstrated that phenazine metabolites are not only essential in fungal pathogen inhibition, but also vital to its competitive fitness in the soil environment.

Pseudomonas aureofaciens strain Tx-1 (Tx-1) was identified and developed at Michigan State University. It is known to produce at least two phenazine antibiotics: PCA and pyrrolnitrin

(Dwyer, 1999). Results of antifungal bioassays demonstrated *in vitro* and *vivo*, PCA, a phenazine metabolite secreted by Tx-1, exhibits antifungal activity against a wide range of turfgrass pathogens identical to that of a chemical fungicide, chlorothalonil (Powell, 2000). This antifungal activity is consistent with the satisfactory control of dollar spot (*R. floccosum*), summer patch (*Magnaporthe poae*), and pink snow mold (*Macrodochrum nivale*) in field studies (Powell, 1993; Dwyer, 1999). After Tx-1 received an EPA label in 2000, a commercial fermentation and irrigation delivery system was developed for it. Adopted by numerous golf courses nationwide, this system allows golf course managers to grow Tx-1 on the course while apply it to the entire golf course through the irrigation system (Dwyer, 1999; Sigler, 2001; Vargas, 2005). Despite its successful introduction to turfgrass disease management, issues with the persistence of Tx-1 on the foliage and its antifungal efficacy under higher disease pressure have arisen. One of the issues related to its persistence and disease suppression in the field is that high inoculum concentrations and high application frequency are needed (Dwyer, 1999). Lower concentration and application frequency result in dramatically decreased disease control (Dwyer, 1999). Inoculums applied through the irrigation system failed to achieve sufficient dollar spot suppression on fairways under intensive disease pressure during summer (Hardebeck, 2004).

Effect of solar ultraviolet radiation (UVR) on microbial biocontrol agents

In most cases, biological control agents are delivered to a ecological unsuitable environment (Deacon, 1991), thus the survival and biological antagonistic activity of biocontrol agents are highly influenced by environmental conditions such as humidity, pH, nutritional availability, temperature, and UV intensity (Lahlali et al., 2011). It has been reported that photo-degradation caused by two different wavelengths of UV in sunlight , UV-A (320-400 nm) and UV-B (280-320 nm), inactivates most microbial insecticides and fungicides under field conditions (Tamez-

Guerra et al., 2005; Hadapad, 2009). Mechanisms of phototoxicity caused by UV-A and UV-B are different. High energy photons that carried by UV-B cause direct damage to DNA by forming cyclobutane pyrimidine dimmers, which disrupt DNA structure and introduce mutation (Pfeifer, 1997; Griffiths, 1998; Jacobs, 2001). Lethal effects triggered by UV-A are mainly due to generation of intracellular reactive oxygen species (Eisenstark, 1987; Griffiths, 1998). The sensitivity to UV has limited the commercial development of certain bioinsecticides (Pusztai M, 1991; Hadapad, 2009), it is reasonable to believe that UV can be more destructive for foliar applied biological control agents (Bull, 1976).

The survival and colonization of bacteria or microbial biocontrol agents in UV intensive habitats, such as the leaf surface, largely depend on tolerance to radiation (Sundin, 2004). Phyllosphere bacteria have developed various strategies to improve UV tolerance (Sundin, 1999), including pigmentation (Sundin, 1999, 2004) and DNA-repair operon (Sundin, 1996). Unlike eukaryotic microbes, whose pigments significantly improve survival under UV-B radiation (Wang, 1994), the small size of bacterial cells limits the utilization of pigment-dependent self-shading mechanisms (Garcia-Pichel, 1994) and may only provide protection against the lower-energy UV-A (Sundin, 2004).

UV sunscreen for microbial biology control agent

Due to the lethal impacts of UV on microbial biocontrol agents in the field, numerous studies have been done in attempt to screen effective UV protectants to improve residual tolerance to UV after application (Tamez-Guerra et al., 2005). Researchers working with biological pesticides, such as *Bacillus sphaericus* Neide, have identified several promising UV protectants (Hadapad, 2009; Lahlali et al., 2011). No UV protectants have ever been tested with

Tx-1 on turf systems. Nevertheless, the discovery of UV protectants for biopesticides prompted a searching for UV protectant candidates for Tx-1.

UV protectant candidate: Humic acid

Humic substances (HS) are produced through the decomposition of organic substances released by dead organisms, a process also called humification (Steinberg, 2004; Trump, 2006).HS is ubiquitous in the environment, and is present in soil, water and sediment (Trump, 2006). Humic acid (HA) is one of the three basic components of HS (Steinberg, 2004; Trump, 2006). Traditionally, HA can be isolated from HS based on its solubility at pH values below pH 2.0 (Steinberg, 2004; Trump, 2006). The potential of using HA as a UV protectant has been supported in multiple papers, in which HA showed the ability to reduce the sensitivity of numerous bacteria and bacteriophages to UV radiation (Corin, 1998; Templeton, 2006; Cantwell, 2008; Lee, 2009). Investigation of the macromolecular structure of HA revealed an abundance of functional groups that contain conjugated unsaturated double bonds capable of absorbing UV radiation (Corin, 1998; Uyguner and Bekbolet, 2005). No inhibition or toxicity was observed when bacteria were grown in HA media (Corin, 1998; Lee, 2009). Effects of HA on turfgrass were studied by Cooper (1998) and Dyke (2009). Their data showed when applied to bentgrass, HA increases root length of bentgrass or root mass.

LITERATURE CITED

Baker, K.F. (1987). Evoloving concepts of biological control of plant pathogens. Annual Review of Phytopathology **25,** 67-85.

Bull, D.L.R., R. L.; House, V. S.; Pryor, N. W. (1976). Improved formulations of the Heliothis nuclear polyhedrosis virus. Journal of Economic Entomology **69,** 731-736.

Cantwell, R., Hofmann, R. and Templeton, M. (2008). Interactions between humic matter and bacteria when disinfecting water with UV light. Journal of Applied Microbiology **105,** 25-35.

Cook, R.J. (1993). Making greater use of introduced microorganisms for biological control of plant pathogens. Annual Review of Phytopathology **31,** 53-80.

Cooper, R.J., Liu, C., Fisher, D.S. (1998). Influence of Humic Substances on Rooting and Nutrient Content of Creeping Bentgrass. Crop Science **38,** 1639.

Corin, N., Backlund, P., Wiklund, T. (1998). BACTERIAL GROWTH IN HUMIC WATERS EXPOSED TO UV-RADIATION AND SIMULATED SUNLIGHT. Chemosphere**,** 1947-1958.

Crouch, J.A., E. N. Weibel, J. C. Inguagiato, et al. . (2003). Suppression of anthracnose on an annual bluegrass putting green with selected fungicides, nitrogen, plant growth regulators, and herbicides. 2003 Rutgers Turfgrass Proceedings **35,** 183-192.

Deacon, J.W. (1991). Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens. Biocontrol Science and Technology **1,** 5-20.

Dwyer, P.J., Jr., . (1999). Field Efficacy, Persistence, and Antibiotic Production of *Pseudomonas auerofaciens.* In Department of Botany and Plant Pathology (East Lansing: Michigan State University).

Dyke, A.V., Johnson, P.G., Grossl, P.R. (2009). Influence of humic acid on water retention and nutrient acquisition in simulated golf putting greens. Soil Use and Management **25,** 255-261.

Eisenstark, A. (1987). Mutagenic and lethal effects of near-ultraviolet radiation (290-400 nm) on bacteria and phage. Environmental and Molecular Mutagenesis **10,** 317-337.

Garcia-Pichel, F. (1994). A model for internal self-shading in planktonic organisms and its implications for the usefulness of ultraviolet sunscreen. Limnol Oceanogr **39,** 1704-1717.

Griffiths, H.R., Mistry, P., Herbert, K.E., Lunec, J. (1998). Molecular and cellular effects of ultraviolet light-induced genotoxicity. Clinical Laboratory Sciences **35,** 189-237.

Hadapad, A.B., Hire, R.S., Vijayalakshmi, N., Dongre, T.K. (2009). UV protectants for the biopesticide based on Bacillus sphaericus Neide and their role in protecting the binary toxins from UV radiation. Journal of Invertebrate Pathology **100,** 147-152.

Hardebeck, G.A., R. F. Turco, R. Latin, and Z. J. Reicher. (2004). Application of Pseudomonas aureofaciens Tx-1 through irrigation for control of dollar spot and brown patch on fairway height turf. HortScience **39(7),** 1750-1753.

Jacobs, J.L.,Carroll, T.L., Sundin, G.W. (2004). The Role of Pigmentation, Ultraviolet Radiation Tolerance, and Leaf Colonization Strategies in the Epiphytic Survival of Phyllosphere Bacteria. Microbial Ecology **49,** 104-113.

Jacobs, J.L., Sundin, G.W. (2001). Effect of Solar UV-B Radiation on a Phyllosphere Bacterial Community. Applied and Environmental Microbiology **67,** 5488-5496.

Kluver, A.J. (1956). Pseudomonas aureofaciens Nov. Spec. and its Pigments. The Journal of Bacteriology **72,** 406-411.

Lahlali, R., Raffaele, B., and Jijakli, M.H. (2011). UV protectants for Candida oleophila (strain O), a biocontrol agent of postharvest fruit diseases. Plant Pathology **60,** 288-295.

Lee, E., Lee, H., Jung, W., Park, S., Yang, D., Lee, K. (2009). Influences of humic acids and photoreactivation on the disinfection of Escherichia coli by a high-power pulsed UV irradiation. Korean Journal of Chemical Engineering **26,** 1301-1307.

Murphy, J., F. Wong, L. Tredway, et al. (2008). Best management practices for anthracnose on annual bluegrass turf. Golf Course Management **76(8),** 93-104.

Nikolai, T.A., Rieke, P.E., Rogers, J.N. III, and Vargas, J.M.Jr. . (2001). Thrfgrass and soil responses to lightweight rolling on putting green root zone mixes. . International Turfgrass Society Research Journal **9,** 604-609.

Palleroni, N.J. (1984). Genus I. Pseudomonas Migula 1894. In Bergey's manual of systematic bacteriology, H.J.G. Krieg N R, ed (Baltimore: The Williams & Wilkins Co.), pp. 141-199.

Pfeifer, G.P. (1997). Formation and processing of UV photoproducts: effects of DNA sequence and chromatin environment. Photochem. Photochemistry and Photobiology **65,** 270-283.

Pierson III, L.S.a.P., E. A. (1996). Phenazine antibiotic production in Pseudomonas aureofaciens: role in rhizosphere ecology and pathogen suppression. FEMS Microbiology Letter **136,** 101-108.

Pierson, L.S.a.L.S.T. (1992). Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30-84. Molecular Plant-Microbe Interactions **5,** 330-339.

Powell, J.F. (1993). Utilization of Bacterial Metabolites for the Management of Fungal Turfgrass Pathogens. In Department of Botany and Plant Pathology (East Lansing: Michigan State University).

Powell, J.F., Vargas, J. M., Jr., et al. (2000). Management of dollar spot on creeping bentgrass with metabolites of Pseudomonas aureofaciens (TX-1). Plant Disease **84,** 19-24.

Pusztai M, F.P., Cringorten L, Kaplan H, Lessard T, Carey PR. (1991). The mechanism of sunlight-mediated inactivation of Bacillus thuringiensis crystals. Biochemical Journal **273,** 43-47.

Putman, A.I., Jung, G., and Kaminski, J. E. (2010). Geographic distribution of fungicideinsensitive Sclerotinia homoeocarpa isolates from golf courses in the northeastern United States. Plant Disease **94,** 186-195.

Sigler, W.V., Nakatsu, C. H., Reicher, Z. J., Turco, R. F. (2001). Fate of the Biological Control Agent Pseudomonas aureofaciens Tx-1 after Application to Turfgrass. Applied and Environmental Microbiology **67,** 3542-3548.

Smiley, R.W., Dernoeden, P. H., and Clarke, B. B. (2005). Compendium of Turfgrass Diseases. (American Phytopathological Society. St. Paul, MN).

Stack, J.P., Kenerley, C. M., and Pettit, R. E. (1988). Biocontrol of Plant Disease. In Application of biological control agents, e. K. G. Mukerju and K. L. Grag, ed (CRC Press, Boca Raton, FL.), pp. 43-54.

Steinberg, C.E.W. (2004). Ecology of Humic Substances in Freshwaters. (Verlag Berlin Heidelberg: Springer).

Sundin, G.W., Jacobs, J.L. (1999). Ultraviolet Radiation (UVR) Sensitivity Analysis and UVR Survival Strategies of a Bacterial Community from the Phyllosphere of Field-Grown Peanut (Arachis hypogeae L.). Microbial Ecology **38,** 27-38.

Sundin, G.W., Kidambi, S.K., Ullrich, M., Bender, C.L. (1996). Resistance to ultraviolet light in *Pseudomonas syringae*: sequence and functional analysis of the plasmid-encoded *rulAB* genes. . Gene **177,** 77-81.

Tamez-Guerra, P., Rodriguez-Padilla, C., and Galán-Wong Luis, J. (2005). Solar Radiation (UV) Protectants for Microbial Insecticides. In Semiochemicals in Pest and Weed Control (American Chemical Society), pp. 127-147.

Templeton, M.R., Hofmann, R., Andrews, R.C. (2006). UV inactivation of humic-coated bacteriohages MS2 and T4 in water. Environmental Engineering Science **5,** 537-543.

Thomashow, L.S., Weller. D. M., Bonsall, R. F. and Pierson. L. S. III. (1990). Production of a phenazine antibiotic by fluorescent Pseudomonas species in the rhizosphere of wheat. Applied and Environmental Microbiology **56,** 908-912.

Towers, G., K. Green, E. Weibel, P. Majumdar and B. B. Clarke. (2003). Evaluation of fungicides for the control of anthracnose basal rot on annual bluegrass. Fungicide and Nematicide Tests **58,** T017.

Trump, J.I.V., Sun, Y., Coates, J.D. (2006). Microbial Interactions with Humic Substances. Advances in Applied Microbiology **60,** 55-96.

Turner, J.M.a.M.A.J. (1986). Occurrence, biochemistry and physiology of phenazine pigment production. Advances in Microbial Physiology **27,** 211-275.

Uyguner, C.S., and Bekbolet, M. (2005). Evaluation of humic acid photocatalytic degradation by UV-vis and fluorescence spectroscopy. Catalysis Today **101,** 267-274.

Vargas, J.M.J. (2005). Management of turfgrass diseases. . (Hoboken, New Jersey: John Wiley & Sons, Inc.).

Wang, Y., Casadavell, A. (1994). Decreased susceptibility of melanized *Cryptococcus neoformans* to UV light. Applied and Environmental Microbiology **60,** 3864-3866.

Weller, D.M. (2007). Pseudomonas biocontrol agents of soilborne pathogents: Looking back over 30 years. Phytopathology**,** 250-256.

Wong, F.P., and S. Midland. (2004). Fungicide resistant anthracnose: bad news for greens management. Golf Course Management **72(6),** 75-80.

CHAPTER ONE

UVR SENSITIVITY TEST FOR TX-1 *IN VITRO* AND MEANS TO IMPROVE ITS UV TOLERANCE

ABSTRACT

A fungicide/antibiotic-producing bacterial strain of *Pseudomonas aureofaciens* Tx-1 (Tx-1) has been commercialized for golf course use with some success in recent years. To achieve adequate control it must be applied daily. This may be partially explained by UV susceptibility of Tx-1, which is a common limitation of biocontrol agents. In this study, results from various wavelengths of ultraviolet radiation exposure tests confirmed this hypothesis by showing a significant drop in Tx-1 survivors following increased dosage. Three approaches were tested to improve UV tolerance of Tx-1: 1) protection with a promising sunscreen, humic acid (HA), was demonstrated in all UV exposure tests. Moreover, addition of HA the highly UV-B sensitive mutant *Pseudomonas aeruginosa* (*phl*$^-$, *uvrA*$^-$) provide statistically significant improvement in UV-B tolerance. 2) In an attempt to identify the most UV-resistant individual cells, Tx-1 was exposed to intensive UV light (UV-C) for various lengths of time (*in vitro*). Cells (10TC30) with significantly improved survival rate after 7.3 J/m^2 UV-C irradiation was found followed by 10 cycles of 42 J/m^2 of UVC exposure. 3) When extend the incubation time to 72 hours, Tx-1 demonstrated better UV-A tolerance at 60 and 90 kJ/m^2. Results from bioassays against *R. floccosum* showed the antifungal activity of Tx-1 did not change after UV exposures.

Introduction

Biocontrol of turfgrass diseases is an important tool for golf course managers. *Pseudomonas aureofaciens* Tx-1 (Tx-1) is a commercially available biocontrol agent used for the control of turfgrass diseases. The persistence of Tx-1 on the foliage and its antifungal efficacy under high disease pressure have been addressed (Dwyer, 1999; Hardebeck, 2004). Preliminary research indicates that the bacterium might be susceptible to inhibition by solar ultraviolet radiation (UVR) (Powell, pers. comm.). Inactivation by UV is a common limitation of biological control agent in the field (Harper, 2006). Photo-degradation caused by two different wavelengths of UVR, UV-A (320-400 nm) and UV-B (280-320 nm), inactivates most microbial insecticides and fungicides in field conditions (Tamez-Guerra et al., 2005; Hadapad, 2009). However, no related data have been reported on UVR sensitivity of Tx-1. Studies have been done in attempt to screen effective UVR sunscreen to improve residual tolerance to UVR after application (Tamez-Guerra et al., 2005). Several promising UVR sunscreen for biological pesticides were identified. A component of humic substances, Humic acid (HA), is a potential UVR sunscreen for Tx-1. It is ubiquitous in the environment (Trump, 2006), has been found to protect microbial organisms from UV radiation (Corin, 1998; Templeton, 2006; Cantwell, 2008), and has low *in vitro* toxicity to microbes. It has been tested on turfgrass before, and no negative effects were observed (Cooper, 1998; Dyke, 2009).

Therefore, we hypothesized that Tx-1 might be sensitive to solar UVR; by promoting the UVR tolerance of Tx-1, it is possible to improve its disease control efficacy in the field. The objectives of this study were to: characterize the sensitivity of Tx-1 to UV-A and UV-B *in vitro* and investigate potential methods for improving UV tolerance of Tx-1. To achieve this goal, UV sensitivity of Tx-1 was first confirmed by challenging it with UV-A and UV-B. In an attempt to

improve UV tolerance of Tx-1, three different approaches were taken: 1) Testing the efficacy of HA as a UV protectant. 2) Promoting pigmentation of Tx-1. 3) Selecting a spontaneously UV-resistant Tx-1 cells. To investigate biocontrol efficacy of Tx-1 with different approaches after UV exposure, *in vitro* bioassays against *R. floccosum* were performed.

Materials and Methods

Preparation of Bacterial Cultures

Tx-1 cultures were grown in 50 mL of Tryptic soy broth (Becton, Dickinson and Company. USA), and *P. aeruginosa* cultures were grown in Luria broth base (LIFE TECHNOLOGIES, Paisley, Scotland) at 25^0C on a rotary shaker at 100 rpm for 24 to 72 hours prior to UV exposure. Before each UV exposure, 1 mL aliquots of the cultures were pelleted and washed twice in 1 mL sterile phosphate buffered saline solution (PBS). These 1 mL cultures were resuspended in 9 mL PBS, or 9 mL PBS plus 2 mL HA stock solution (10 mg/mL) (SIGMA-ALDRICH, Co., Spruce Street, St. Louis, MO) in a sterile glass petri dish and stored on ice until irradiation.

UV irradiation

Cultures were exposed to different UV lamps: UV-B wavelengths were generated by an XX-15M UV lamp (UVP Products, San Gabriel, CA.) and filtered through polystyrene which blocks UV-C wavelengths (<290 nm) (Sundin, 2001); UV-A wavelengths was generated by an XX-15L; UV-C radiation was produced by an XX-15C UV lamp (UVP Products). The lamps were fixed at constant height above the bacterial cultures. UV output was measured by a UV-X radiometer (UVP Products) once lamps were warmed up and stabilized for 15 min. Energy intensity of all UV wavelengths was determined based on UV output levels, as used in previous

studies (Jacobs, 2001; Sundin, 2004). Bacterial cultures were constantly agitated by a rocking shaker (seven revolutions per minute) under the UV-A lamp or by hand under UV-B or UV-C lamps to prevent shading. To avoid light repair mechanisms, all bacterial cultures were kept in a dark condition during UV-B and UV-C irradiations.

After each UV irradiation, 200-µl samples were transferred from the dish to sterile micro-tubes for dilution. Cultures were serially diluted in sterile PBS to 10^{-5}, 10^{-6} or 10^{-7} concentrations by repeatedly transferring 40-µl samples into 360 µl PBS until the targeted dilution concentration was reached. Three to four replicates of 50 µl of diluted samples were then plated on 0.5 X potato dextrose agar (Becton, Dickinson and Company. Sparks, MD) plates for Tx-1, or Luria agar for *P. aeruginosa* (Becton, Dickinson and Company. Sparks, MD). All plates from UV-B and UV-C irradiations were then incubated for 72 h at 25^{0}C under dark conditions.

After incubation, plates were examined and bacterial colonies counted. The mean CFU/0.05 ml was calculated by multiplying the number of CFUs by the dilution factor.

Effects of Humic acid on population size of Tx-1

To exam the affects of HA on Tx-1, bacterial cultures were grown in 100 mL of Tryptic soy broth (Becton, Dickinson and Company. Sparks, MD) mixed with 0.2 g of HA sodium salt (SIGMA-ALDRICH, Co. St. Louis, MO). pH of the growth media and population size were monitored up to 24 hours.

Bioassay against dollar spot fungus

Bacteria were grown on potato dextrose agar (Becton, Dickinson and Company. Sparks, MD) for 48 hours at 25^0C. One transfer loop was streaked down the center of each PDA plate. The plates were incubated at 25^0C for 24 hours prior to fungal inoculation. *Rutstroemia floccosum* (dollar spot), was grown on PDA. Four 2-mm agar plugs containing fungal mycelia were transferred to two PDA plates without bacteria (control) and two plates with *P. aureofaciens*. Each plug represented one replication. Plates were incubated at 25^0C. Fungal growth was measured after two days of incubation. Colony diameter was measured parallel to and perpendicular to the bacterial streak, and the average radius was calculated from these measurements. Colony area was calculated as πr^2, where r = average radius.

Data analysis

Data was analyzed by using the SAS (Statistical Analysis Software, Cary, N.C.) ANOVA least significant difference (LSD) test (p=0.05). All tests were replicated three times.

Results

Characterization of the solar UVR sensitivity for Tx-1

To estimate the solar UV sensitivity of Tx-1, the bacterium was exposed to UV-A or UV-B. The exposure length of UV irradiations was per-determinate based on average of four hour (1100 to 1500) solar UV irradiance during summer time (Sundin, 2004). The sensitivity to UV-A and UV-B was compared with unexposed cells. Results showed a marked reduction in UV-A survival following doses of 60 kJ/m^2 and 90 kJ/m^2 (Figure 1). Percentage of survival decreased significantly to 41.6% when the dose increased to 120 kJ/m^2. After received 180 kJ/m^2 UV-A, up to 94% cells were killed (Figure 1.01). Results from UV-B exposure showed survival

significantly dropped to 8.9 % after 8.26 kJ/m^2 dose of UV-B (Figure 1.02). Only 4% survived 13.77 kJ/m^2 UV-B exposure, which is close to the average UV-B output within 4 h during summer time (Sundin, 2004) (Figure 1.02). Together, this observation suggests that Tx-1 is sensitive to solar UV radiation.

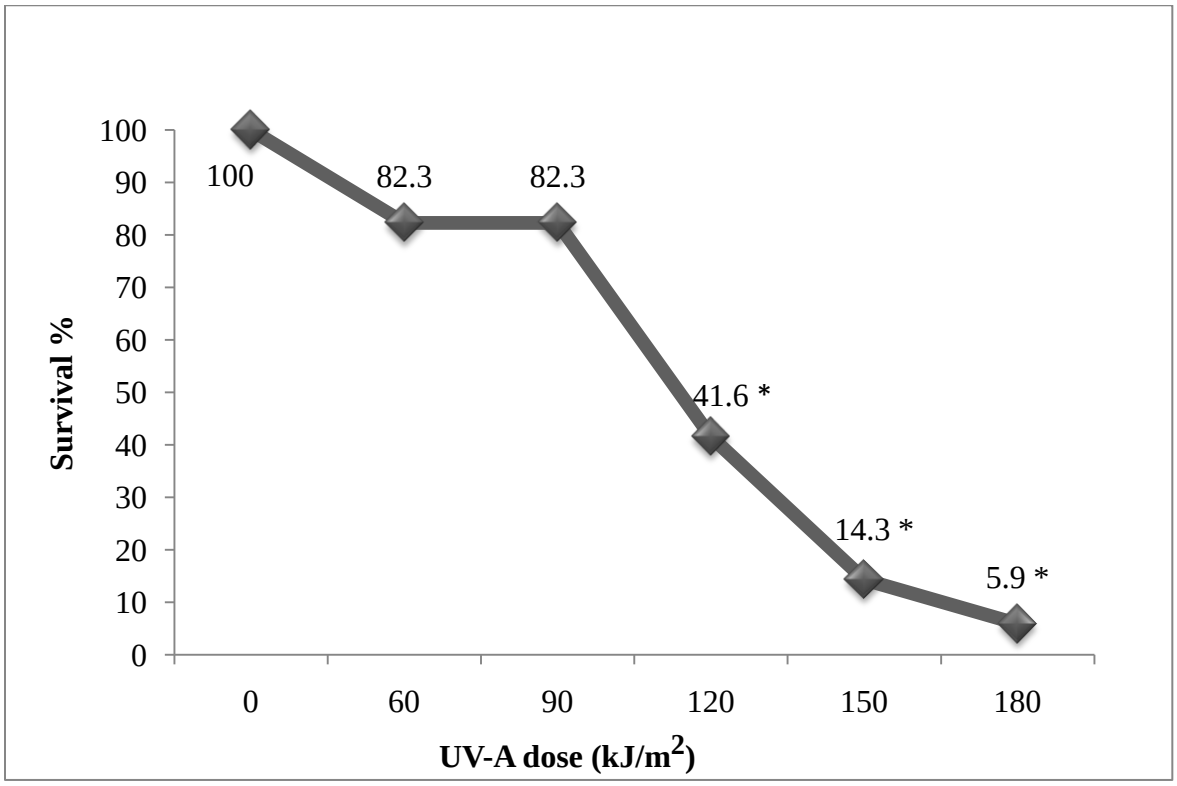
UVR protection from humic acid (HA)

The objective of these experiments was to determine the effect of HA on UVR degradation on Tx-1. In this study, HA was added with bacterial cultures as described in material and methods. The culture + HA mixture was then challenged with UV-A, UV-B or UV-C. UVR protection from HA was demonstrated in both UVR exposure tests (Figure 1.03-1.05). Compared to unprotected Tx-1, which suffered a significant drop in survivors following increased UVR doses as described in previous section, up to 100% and 77% of Tx-1 were protected by addition of HA when the dose increased to 180kJ/m^2 of UV-A and 13.77 kJ/m^2 of UV-B, respectively (Figure 1.03, 1.04). When exposed to high-energy UV-C wavelengths, 1- to 4-log more inactivation of unprotected Tx-1 was observed for UVR doses ranged from 7.1 to 42.6 J/m^2 (Figure 1.05), which is consistent with the sensitivity to UV-A and UV-B, while 85% Tx-1 survived with the protection of HA.

In previous tests on HA, 120 mg/L HA demonstrated the best protection for UV sensitive bacteria under 14 mJ/cm^2 (0.14kJ/m^2) UV exposure (Cantwell, 2008). Due to the intensiveness of UV radiation level in our tests, concentration of HA was set to 1.67g/L, approximately 100 times more than 120 mg/L. To determine the optimal concentration of HA for UV protection, a highly UV-B sensitive bacterium, *Pseudomonas aeruginosa* (phl^-, $uvrA^-$), was tested. The

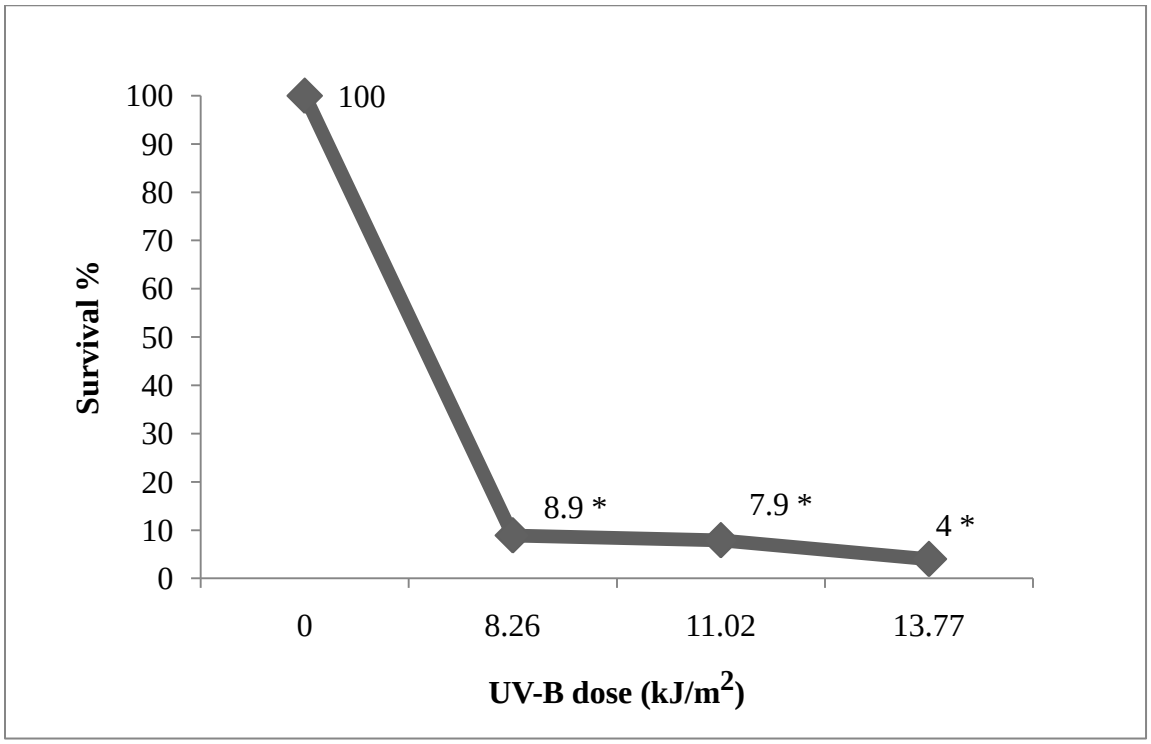
addition of HA conferred UV-B tolerance to this highly susceptible *P. aeruginosa* mutant (Table 1.01). Unprotected cells died with 0.2 kJ/m^2 of exposure while 66.3% of cultures that were mixed with HA survived. When the UV dose increased to 0.9 J/m^2, 23% of *P. aeruginosa* culture survived with the addition of HA. Four different concentrations of HA (1.67g/L, 0.89g/L, 0.42g/L and 0.17g/L) were tested with *P. aeruginosa*. Results show that the addition of 1.67g/L HA significantly improved UV tolerance of Tx-1 during the 10 min UV-B exposure, while 0.89g/L HA was unable to protect the cells when the energy of UV-B increased to 0.9 kJ/m^2 (Table 1.02). Collectively, the addition of HA significantly increased UV tolerance of Tx-1 and the *P. aeruginosa* UV-B sensitive mutant.

Figure 1.01 Survival of *P. aureofaciens* Tx-1 after exposed to UV-A radiation.



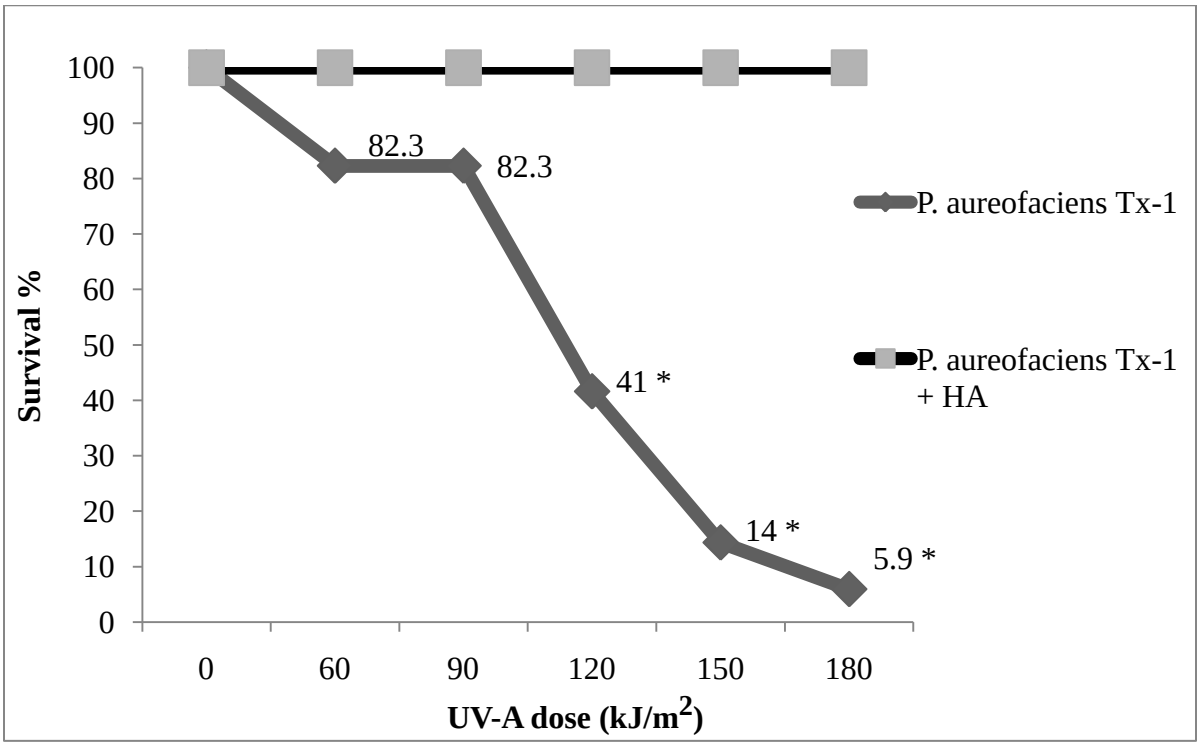
*, Significant at the 0.05 statistical probability level.

Figure 1.02 Survival of *P. aureofaciens* Tx-1 after exposed to UV-B radiation.



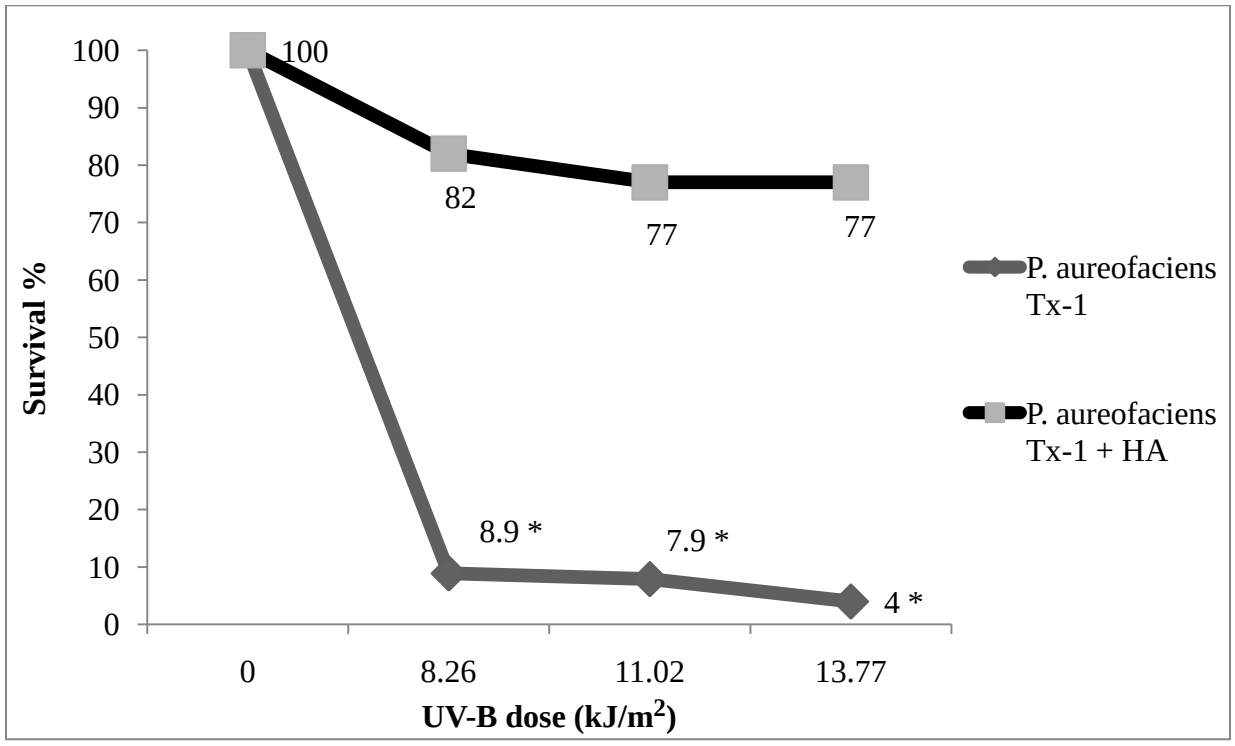
*, Significant at the 0.05 statistical probability level.

Figure 1.03. Survival of *P. aureofaciens* Tx-1 with or without the addition of humic acid after exposed to UV-A.



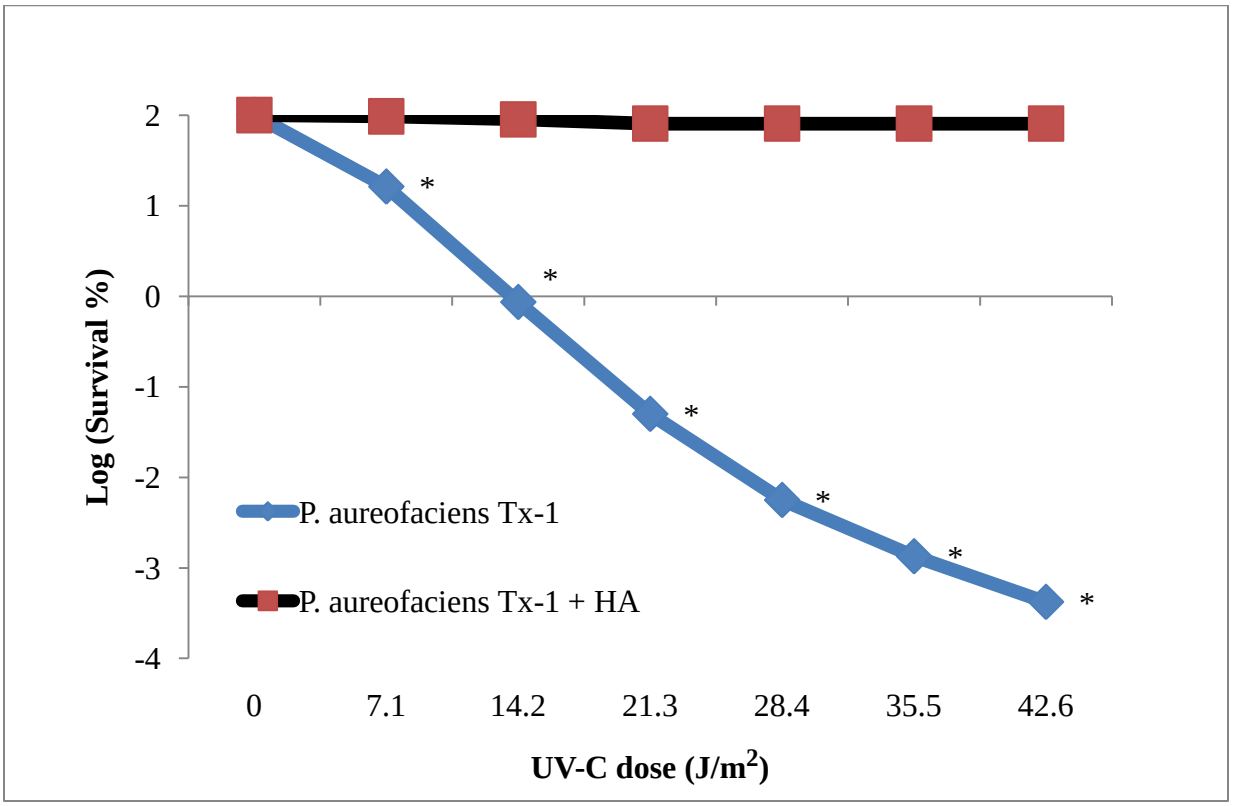
*, Significant at the 0.05 statistical probability level.

Figure 1.04. Survival of *P. aureofaciens* Tx-1 with or without the addition of humic acid after exposed to UV-B.



*, Significant at the 0.05 statistical probability level.

Figure 1.05. Log survival of *P. aureofaciens* Tx-1 with or without the addition of humic acid after exposed to UV-C radiation.



*, Significant at the 0.05 statistical probability level.

Table 1.01. Survival of *P. aeruginosa (phr-, uvrA-)* or *P. aureofaciens* Tx-1 humic acid mixture after exposed to 1.69 J m^{-2} s^{-1} UV-B radiation.

Treatment	Time (min)	Percentage of survival	LSD[a]
P. aeruginosa (phr-, uvrA-)	0	100	a
P. aeruginosa (phr-, uvrA-) +1.67g/L HA	0	100	a
P. aeruginosa (phr-, uvrA-) +1.67g/L HA	2.5	82.6	b
P. aeruginosa (phr-, uvrA-) +1.67g/L HA	5	34.8	c
P. aeruginosa (phr-, uvrA-) +1.67g/L HA	10	26.1	cd
P. aeruginosa (phr-, uvrA-)	2.5	0	d
P. aeruginosa (phr-, uvrA-)	5	0	d
P. aeruginosa (phr-, uvrA-)	10	0	d

[a], Treatment means followed by the same letter do not significantly differ (LSD, p=0.05).

Table 1.02. Survival of *P. aeruginosa (phr-, uvrA-)* or *P. aeruginosa (phr-, uvrA-)* mixed with different concentrations of humic acid after exposed to 1.49 J m^{-2} s^{-1} UV-B radiation.

Treatment	UV-B dose (kJ/m^2)	Percentage of survival	LSD [a]
P. aeruginosa (phr-, uvrA-)+ 0.89g/L HA	0	100.00	a
P. aeruginosa (phr-, uvrA-)+ 0.42g/L HA	0	100.00	a
P. aeruginosa (phr-, uvrA-)	0	100.00	a
P. aeruginosa (phr-, uvrA-)+ 0.17g/L HA	0	100.00	a
P. aeruginosa (phr-, uvrA-)+ 1.67g/L HA	0	100.00	a
P. aeruginosa (phr-, uvrA-)+ 1.67g/L HA	0.25	66.34	b
P. aeruginosa (phr-, uvrA-)+ 1.67g/L HA	0.5	49.71	c
P. aeruginosa (phr-, uvrA-)+ 0.89g/L HA	0.25	31.11	d
P. aeruginosa (phr-, uvrA-)+ 1.67g/L HA	1.04	22.97	e
P. aeruginosa (phr-, uvrA-)+ 0.89g/L HA	0.5	11.89	f
P. aeruginosa (phr-, uvrA-)+ 0.89g/L HA	1.04	1.79	g
P. aeruginosa (phr-, uvrA-)+ 0.42g/L HA	0.25	1.17	h
P. aeruginosa (phr-, uvrA-)	0.25	0.00	h
P. aeruginosa (phr-, uvrA-)+ 0.17g/L HA	0.25	0.00	h
P. aeruginosa (phr-, uvrA-)	0.5	0.00	h
P. aeruginosa (phr-, uvrA-)+ 0.17g/L HA	0.5	0.00	h
P. aeruginosa (phr-, uvrA-)+ 0.42g/L HA	0.5	0.00	h
P. aeruginosa (phr-, uvrA-)	1.04	0.00	h
P. aeruginosa (phr-, uvrA-)+ 0.17g/L HA	1.04	0.00	h
P. aeruginosa (phr-, uvrA-)+ 0.42g/L HA	1.04	0.00	h

[a], Treatment means followed by the same letter do not significantly differ (LSD, p=0.05).

Table 1.03. Average fungal colony area (cm^2) of *R. floccosum* on PDA medium inoculated with *P. aureofaciens* Tx-1 or *P. aureofaciens* Tx-1 humic acid mixture that received 90-180 kJ/m^2 UV-A radiation.

		2 DAT		4 DAT	
	UV-A Dose (kJ/m^2)	Average fungal colony area(cm^2)[b]	LSD[a]	Average fungal colony area(cm^2)[b]	LSD[a]
P. aureofaciens Tx-1	0.00	2.2	cd	6.2	b
P. aureofaciens Tx-1	90.00	1.9	cd	4.6	bc
P. aureofaciens Tx-1	180.00	6.5	b	5.2	b
P. aureofaciens Tx-1+ humic acid	0.00	1.7	cd	4.5	bc
P. aureofaciens Tx-1+ humic acid	90.00	1.2	d	2.4	c
P. aureofaciens Tx-1+ humic acid	180.00	1.7	cd	4.2	bc
Unexposed control	0.00	15.7	a	29.7	a

[b] ,Average fungal colony area (cm^2) calculated using πr^2, where r=average radius.

[a], Treatment means followed by the same letter do not significantly differ (LSD, p=0.05).

Table 1.04. Average fungal colony area (cm^2) of *R. floccosum* on PDA medium inoculated with *P. aureofaciens* Tx-1 or *P. aureofaciens* Tx-1 humic acid mixture that received 20.85-41.7 J/m^2 UV-C radiation.

		2 DAT		4 DAT	
	UV-C Dose (J/m^2)	Average fungal colony area(cm^2) [b]	LSD[a]	Average fungal colony area(cm^2) [b]	LSD[a]
P. aureofaciens Tx-1	0.00	1.8	c	4.4	b-d
P. aureofaciens Tx-1	20.85	0.8	e	1.8	f
P. aureofaciens Tx-1	41.7	1.2	c-e	2.7	d-f
P. aureofaciens Tx-1+ humic acid	0.00	1.6	cd	4.8	bc
P. aureofaciens Tx-1+ humic acid	20.85	1.3	c-e	3.8	c-e
P. aureofaciens Tx-1+ humic acid	41.7	0.9	de	2.2	ef
Unexposed control	0.00	15.7	a	29.7	a

[b], Average colony area (cm^2) calculated using πr^2, where r=average radius.

[a], Treatment means followed by the same letter do not significantly differ (LSD, p=0.05).

Table 1.05. Average fungal colony area (cm^2) of *R. floccosum* on PDA medium inoculated with *P. aureofaciens* Tx-1 or *P. aureofaciens* Tx-1 humic acid mixture that received 8.26-13.77 kJ/m^2 UV-B radiation.

		1 DAT		2 DAT		4 DAT	
Treatment	UV-B dose (kJ/m^2)	Average fungal colony area(cm^2) [b]	LSD[a]	Average fungal colony area(cm^2) [b]	LSD[a]	Average fungal colony area(cm^2) [b]	LSD[a]
P. aureofaciens Tx-1+ humic acid	0	1	c	2.52	b	5.7	b
P. aureofaciens Tx-1+ humic acid	11.02	1.1	c	2.81	b	6.0	b
P. aureofaciens Tx-1+ humic acid	13.77	1.2	bc	2.77	b	5.9	b
P. aureofaciens Tx-1	8.26	1.2	bc	3.21	b	6.1	b
P. aureofaciens Tx-1+ humic acid	8.26	1.3	bc	2.55	b	5.4	b
P. aureofaciens Tx-1	11.02	1.4	bc	2.69	b	5.5	b
P. aureofaciens Tx-1	0	1.5	bc	2.87	b	5.7	b
P. aureofaciens Tx-1	13.77	1.7	b	3.17	b	6.2	b
Control	0	4.8	a	18.1	a	29.9	a

[b], Average colony area (cm^2) calculated using πr^2, where r=average radius.

[a], Treatment means followed by the same letter do not significantly differ (LSD, p=0.05).

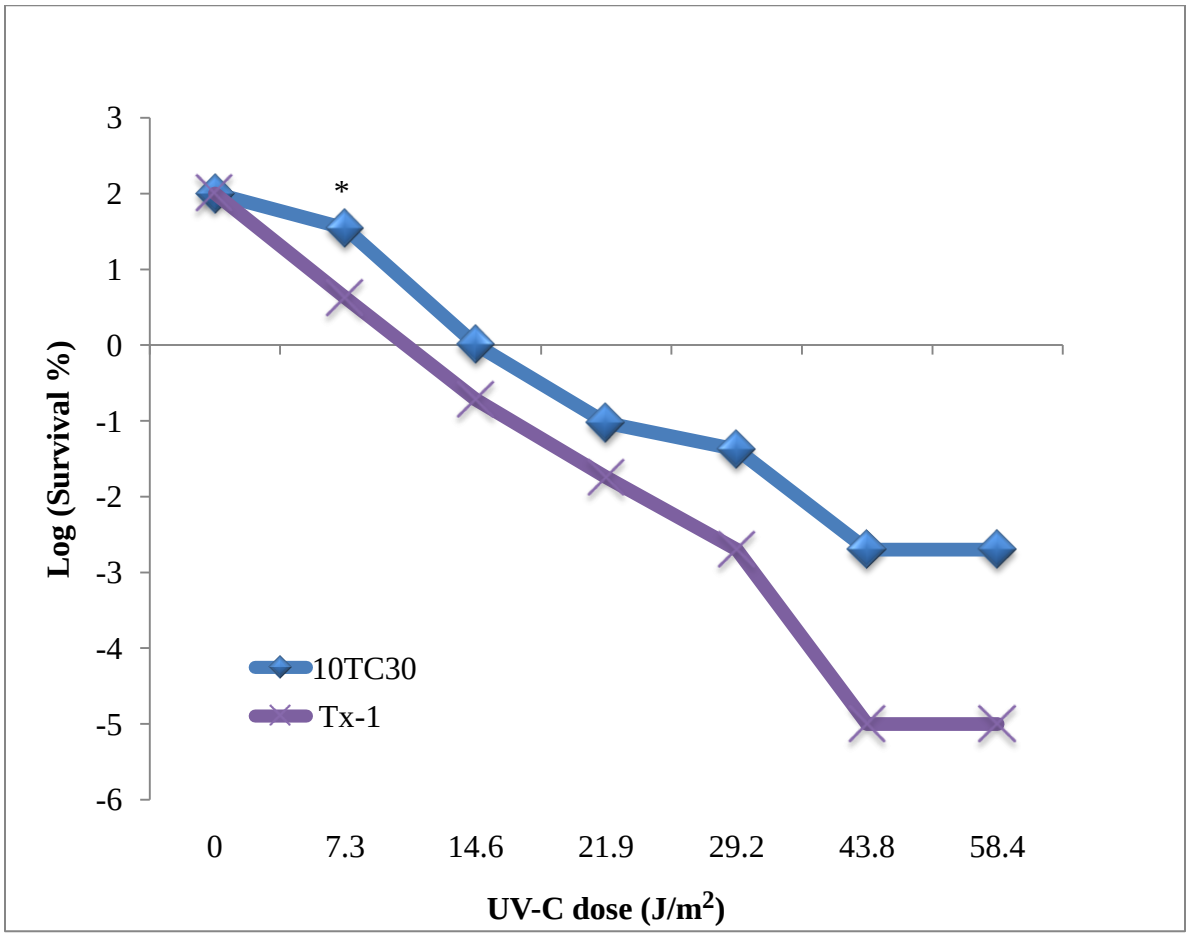
In vitro bioassay tests

To investigate the anti-fungal property of Tx-1 after it was exposed to UV or mixed of HA, *in vitro* bioassays against *R. floccosum* (dollar spot) were performed as described in material and methods. Results showed the biological control efficacy of Tx-1 cells that received UVR exposure, with or without HA addition against *R. floccosum* pathogen was maintained compared to unexposed Tx-1 (Table 1.03-1.05).

Identification of improved UVR tolerant bacterial strains of Tx-1

UV-C wavelengths introduce similar yet more intense DNA damage to microbes (Sundin, 1999). This wavelengths have been used to generate data to estimate the UV-B tolerance of close related bacteria (Sundin, 2004). In an attempt to identify UVR tolerant strains, cells of Tx-1 were exposed to multiple cycles of 42 J/m^2 of UV-C radiation as described in material and methods. Survivors of Tx-1 after each radiation cycle were selected and exposed to 42 J/m^2 of UV-C radiation along with the unexposed Tx-1. The strain of Tx-1, which survived through 10 cycles of 42 J/m^2 of UV-C exposures, was selected and named as 10TC30. 30% more 10TC30 cells survived after exposure to 7.3 J/m^2 of UV-C compared to unexposed Tx-1 (Figure 1.06). When exposed to UV-A, 10TC30 showed no difference in survival compared to unexposed Tx-1 (Table 1.06).

Figure 1.06. Log survival of *P. aureofacious* Tx-1 and 10TC30 after exposed to UV-C radiation.



*, Significant at the 0.05 statistical probability level.

Table 1.06. Survival of *P. aureofaciens* Tx-1 or 10TC30 strain after exposed to 60-180 J/m^2 UV-B radiation.

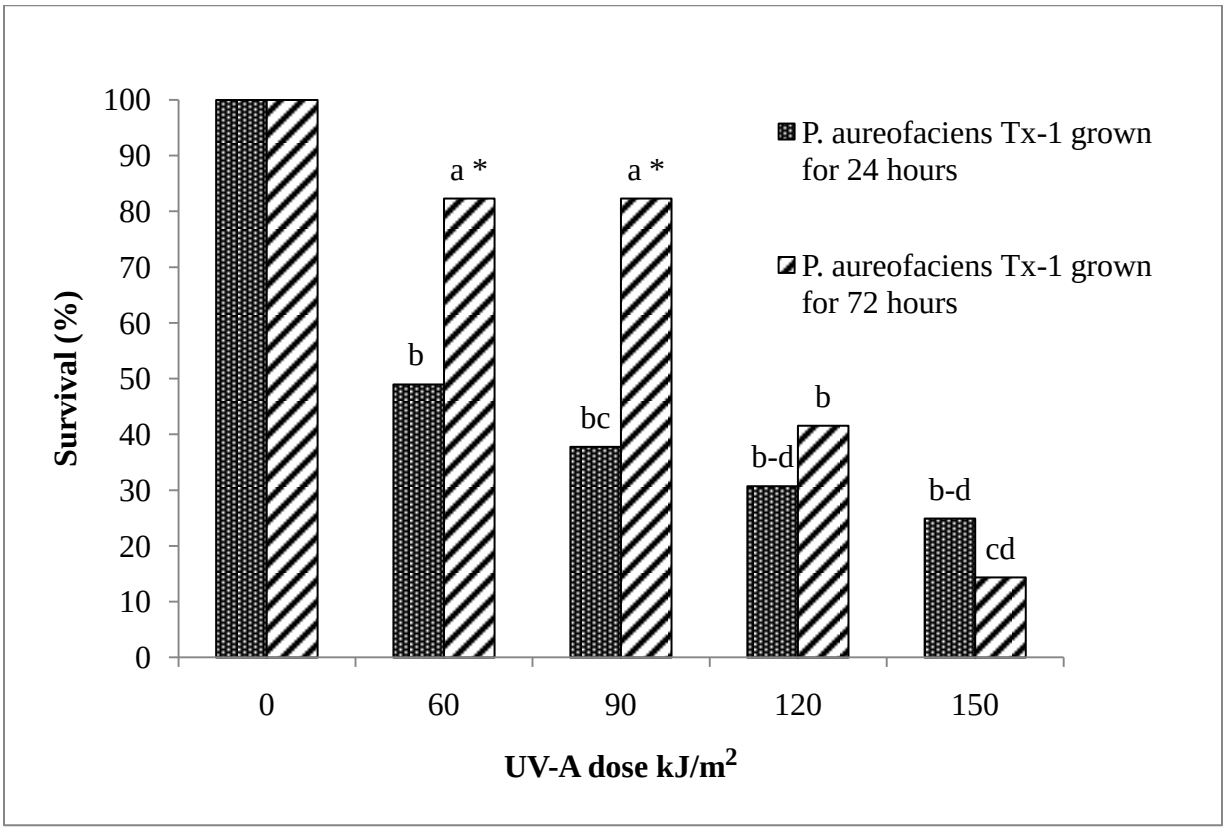
Treatment	UV-A Dose (kJ/m^2)	Percentage of survival	LSD[a]
P. aureofaciens Tx-1	0	100	ab
P. aureofaciens Tx-1	60	100	ab
P. aureofaciens Tx-1	90	63.103	c
P. aureofaciens Tx-1	120	51.724	cd
P. aureofaciens Tx-1	150	46.207	de
P. aureofaciens Tx-1	180	31.034	ef
10TC30	0	100	ab
10TC30	60	85.556	b
10TC30	90	67.222	c
10TC30	120	37.778	d-f
10TC30	150	41.667	d-f
10TC30	180	28.333	f

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

Prolonging incubation time to increase UVR tolerance

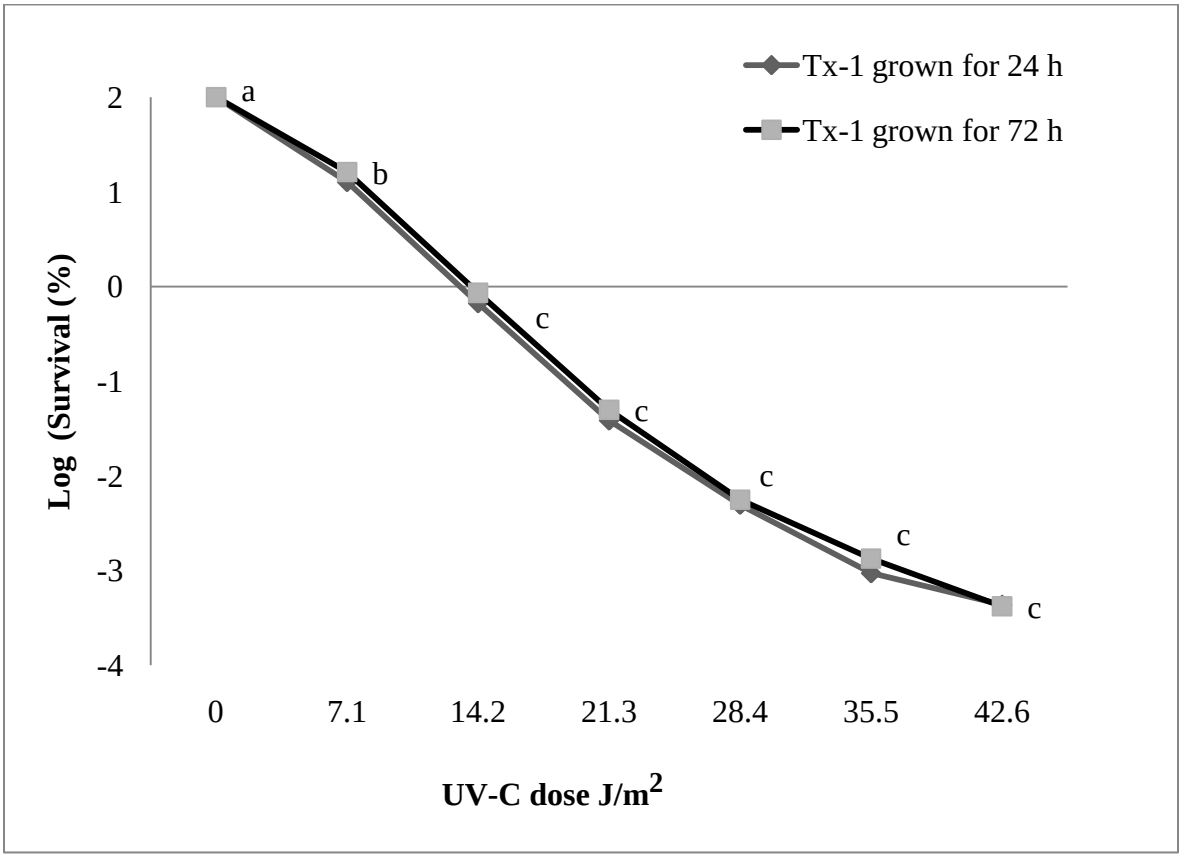
Tx-1 cultures that incubated for 24 or 72 h were tested for sensitivity to UV. The effects of increased incubation period which produce more pigment in the cells were tested to see if the pigment increases TX-1 tolerance to UV exposure. Tx-1 cultures incubated for 72 hours performed significantly better when exposed to less than 90 kJ/m^2 UV-A irradiation than did the 24 hours cultures (Figure 1.07). There were no differences in the survival cells from either incubation periods when the energy output of UV-A was above 90 kJ/m^2 (Figure 1.07). This improved UV tolerance of Tx-1 due to prolonged incubation time was not consistent under UV-C exposure, since no difference were observed between these two incubation time after UV-C exposures (Figure 1.08).

Figure 1.07. Survival of *P. aureofacious* Tx-1 incubated for 24 or 72 hr. after exposed to UV-A radiation.



*, Means followed by the same letter are not significantly different according to Fisher's LSD (P>0.05).

Figure 1.08. Log survival of *P. aureofacious* Tx-1 after UV-C exposure.



Means followed by the same letter are not significantly different according to Fisher's LSD (P>0.05).

Discussion

This is the first analysis of UVR sensitivity test for *P. aureofacious* Tx-1, and the results confirmed that the UV-A and UV-B wavelengths within sunlight can largely limited the survival of Tx-1. More than 90% of Tx-1 cells were killed by UV output which is close to 4 hr of solar UV during the summer time (Figure 1.01, 1.02). This is not surprising, since solar photo-inhibition has been shown to inactivate most microbial biocontrol agents in the field (Tamez-Guerra et al., 2005; Hadapad, 2009). Interestingly, although the survival of Tx-1 was significantly decreased after UV radiation, results from bioassay show no change in anti-fungal activity against *R. floccosum* (Table 1.03-1.05). It could be possible that the cellular damage caused by UVR did not affect the process of antibiotic production of Tx-1. This observation indicates that selection for UV tolerant isolates could be made. The UV sensitivity of Tx-1 addresses the necessity of developing strategies to improve UV tolerance of Tx-1.

One way to improve UV tolerance of biocontrol agent is through adding sunscreen materials (Tamez-Guerra et al., 2005). The potential of using HA as a UV protectant has been supported in multiple papers, in which HA showed the ability to reduce the sensitivity of numerous bacteria and bacteriophages to UV radiation (Corin, 1998; Templeton, 2006; Cantwell, 2008). Different from these studies, in which HA was tested only under low-dose UV radiation (0.05-0.14 kJ/m^2), this is the first study to demonstrate the UV protection property of HA under intensive UV-A (60-180 kJ/m^2), UV-B (8.26-13.77 kJ/m^2), and UV-C (7.1-42.6 J/m^2) radiation (Figure 1.03-1.05). It has been suggested that the degree of UV protection from HA is concentration dependent. This was demonstrated when a highly UV-B sensitive *Pseudomonas aeruginosa* (*phl*$^-$, *uvrA*$^-$) strain was mixed with various concentration of HA (Cantwell, 2008). Our data are in the agreement with this hypothesis by demonstrating the level of UV protection

from HA dropped significantly as the concentration decreased (Table 1.02). The fact that anti-fungal activity of Tx-1 was not changed after mixing with HA further supports the role of HA as a suitable UV sunscreen for Tx-1.

Based on the results from UV sensitivity tests of Tx-1, which demonstrate unchanged anti-fungal activity of Tx-1 following UV-A, UV-B, and UV-C exposures (Table 1.03-1.05), we hypothesized that by using intensive UV radiation, it is possible to isolate UV tolerant Tx-1 without affecting anti-fungal property. 10TC30, a strain of Tx-1 which survived 10 cycles of 42 J/m^2 of UV-C exposures, showed significantly improved UV tolerance (Figure 1.06). This result is similar to the recent studies which demonstrate UVR-inducible mutability can lead to gains in UVR tolerance (Weigand, 2009, 2011). We hypothesize that the increase in UVR tolerance of Tx-1 after receiving multiple cycles of UV-C exposure could be a result of the inducible mutability which was trigged by UV-C. When exposed under UV-A, 10TC30 showed no increasing in UV-A tolerance (Table 1.06). This is not a surprise, since the mechanisms of photo-toxicity caused by UV-A are different from DNA-damaging UV-B/UV-C (Jacobs, 2001; Sundin, 2004). Moreover, it has been shown that UV-B/UV-C tolerance may not translate to UV-A tolerance (Sundin, 2004). More tests are needed to understand the nature of UVR tolerance of 10TC30.

It has been proposed that cell pigmentation could increase UV-A tolerance (Sundin, 2004). Tx-1 is known to produce at least one pigmented antibiotic, PCA (Dwyer, 1999). The production of the antibiotics of Tx-1 occur primarily during the stationary phase, which starts at about 20 h of incubation (Powell, 1993). Hence, we hypothesized that prolonged incubation period may promote the production of PCA, which may lead to an improvement in UV-A

tolerance. Indeed, Tx-1 incubated for 72 h was more tolerant to UV-A (Figure 1.07), but the sensitivity to UV-C radiation of Tx-1 from both incubation times remained the same (Figure 1.08). Thus, the increased incubation period is most likely to increase only UV-A tolerance of Tx-1. It is important to note that the population size of the longer incubation period was 1 log larger than the 24 h one. More UV energy is needed to inactive bacteria as the population increased (Gomes, 2008). The improvement in population size of Tx-1 may also contribute to its UV-A tolerance.

LITERATURE CITED

Cantwell, R., Hofmann, R. and Templeton, M. (2008). Interactions between humic matter and bacteria when disinfecting water with UV light. Journal of Applied Microbiology **105,** 25-35.

Cooper, R.J., Liu, C., Fisher, D.S. (1998). Influence of Humic Substances on Rooting and Nutrient Content of Creeping Bentgrass. Crop Science **38,** 1639.

Corin, N., Backlund, P., Wiklund, T. (1998). BACTERIAL GROWTH IN HUMIC WATERS EXPOSED TO UV-RADIATION AND SIMULATED SUNLIGHT. Chemosphere**,** 1947-1958.

Dwyer, P.J., Jr., . (1999). Field Efficacy, Persistence, and Antibiotic Production of *Pseudomonas auerofaciens.* In Department of Botany and Plant Pathology (East Lansing: Michigan State University).

Dyke, A.V., Johnson, P.G., Grossl, P.R. (2009). Influence of humic acid on water retention and nutrient acquisition in simulated golf putting greens. Soil Use and Management **25,** 255-261.

Gomes, A.I., Santos, J.C., Vilar, V.J.P., Boaventura, R.A.R. (2008). Inactivation of Bacteria *E. coli* and photodegradation of humic acids using natural sunlight. Applied Catalysis B: Environmental **88,** 283-291.

Hadapad, A.B., Hire, R.S., Vijayalakshmi, N., Dongre, T.K. (2009). UV protectants for the biopesticide based on Bacillus sphaericus Neide and their role in protecting the binary toxins from UV radiation. Journal of Invertebrate Pathology **100,** 147-152.

Hardebeck, G.A., R. F. Turco, R. Latin, and Z. J. Reicher. (2004). Application of Pseudomonas aureofaciens Tx-1 through irrigation for control of dollar spot and brown patch on fairway height turf. HortScience **39(7),** 1750-1753.

Harper, D.R. (2006). Biological contorl by microoorganisms. In Encyclopedia of Life Sciences (John Wiley & Sons.

Jacobs, J.L., Sundin, G.W. (2001). Effect of Solar UV-B Radiation on a Phyllosphere Bacterial Community. Applied and Environmental Microbiology **67,** 5488-5496.

Jacobs, J.L., Carroll, T.L., Sundin, G.W. (2004). The Role of Pigmentation, Ultraviolet Radiation Tolerance, and Leaf Colonization Strategies in the Epiphytic Survival of Phyllosphere Bacteria. Microbial Ecology **49,** 104-113.

Kim, J.J., Sundin, G.W. (2001). Construction and Analysis of Photolyase Mutants of *Pseudomonas aeruginosa* and *Pseudomonas syringae*: Contribution of Photoreactivation, Nucleotide Excision Repair, and Mutagenic DNA Repair to UV-B Radiation. Applied and Environmental Microbiology **67,** 1405-1411.

Powell, J.F. (1993). Utilization of Bacterial Metabolites for the Management of Fungal Turfgrass Pathogens. In Department of Botany and Plant Pathology (East Lansing: Michigan State University).

Sundin, G.W., Jacobs, J.L. (1999). Ultraviolet Radiation (UVR) Sensitivity Analysis and UVR Survival Strategies of a Bacterial Community from the Phyllosphere of Field-Grown Peanut (Arachis hypogeae L.). Microbial Ecology **38,** 27-38.

Tamez-Guerra, P., Rodriguez-Padilla, C., and Galán-Wong Luis, J. (2005). Solar Radiation (UV) Protectants for Microbial Insecticides. In Semiochemicals in Pest and Weed Control (American Chemical Society), pp. 127-147.

Templeton, M.R., Hofmann, R., Andrews, R.C. (2006). UV inactivation of humic-coated bacteriohages MS2 and T4 in water. Environmental Engineering Science **5,** 537-543.

Trump, J.I.V., Sun, Y., Coates, J.D. (2006). Microbial Interactions with Humic Substances. Advances in Applied Microbiology **60,** 55-96.

Weigand, M.R., Sundin, G.W.,. (2009). Long-term effects of inducible mutagenic DNA repair on relative fitness and phenotypic diversification in *Pseudomonas cichorii* 302959. Genetics **181,** 199-208.

Weigand, M.R., Tran, V.N., Sundin, G.W.,. (2011). Growth Paraeter Components of Adaptive Specificity during Experimental Evolution of the UVR-Inducible Mutator *Pseudomonas cichorii* 302959. PUBLIC LIBRARY of SCIENCE One **6,** e15975.

CHAPTER TWO

FIELD STUDY FOR TX-1, 10TC30, AND HUMIC AICD

ABSTRACT

To understand the effect of improved UV tolerance on the disease control efficacy under field condition, a study was done in July to October of 2009 and 2010. Various concentrations of Tx-1 or 10TC30, along with combinations with and without HA were tested for disease control. When applied at 10^7 CFU/cm^2, Tx-1 and Tx-1 + HA treatments resulted in similar dollar spot and anthracnose suppression. When applied at 10^5 CFU/cm^2, Tx-1 alone treatment provided significant dollar spot control at six rating dates in 2009, while Tx-1 + HA showed no effect on dollar spot incidence. HA alone treatment showed no effect on disease severity and turf quality for over two years. 10TC30 applied at 10^7 CFU/cm^2 was the first biological treatment that significantly controlled dollar spot on 21-Jul 2010. Results for population sampling showed that the 10TC30 + HA treatment had significantly higher log population size than Tx-1 alone treatment. All together, these results indicate that the addition of HA has limited effect on disease control efficacy of Tx-1. The improvement in only UV tolerance might not be conclusive for promoting the overall persistence and disease control efficacy of Tx-1.

Introduction

The ability to persist and colonize in the environment at sites when disease control is needed is a requirement for an effective biological control in the field (Chang, 1981). In most cases, the inability to persist or establish itself in a specific crop system leads to the failure of this potential biological control agent (Wood, 1996). It has been shown that photo-degradation caused by two different wavelengths of UV, UV-A (320-400 nm) and UV-B (280-320 nm), inactivates most microbial insecticides and fungicides in field conditions (Tamez-Guerra et al., 2005; Hadapad, 2009). The results from *in vitro* studies confirmed the UVR susceptibility of Tx-1 by showing a significant decrease in Tx-1 survivors following increased UV dosage. To improve the UVR tolerance of Tx-1, three methods were tested: 1) a potential UV sunscreen, humic acid (HA) was found and showed significant protection for Tx-1 in all UV exposure tests. 2) 10TC30, the strain of Tx-1 which survived 10 cycles of 30 sec of UV-C exposure was identified and showed improved tolerance to 7.3 J/m^2 UV-C irradiation. 3) Prolonging the incubation period from 24 to 72 hr demonstrated better UV-A tolerance at 60 and 90 kJ/m^2. Moreover, results from bioassays against *R. floccosum* showed the production of efficacious antibiotics did not change after UV exposures or the addition of HA.

Disease control efficacy under field condition is the essential test for any disease management agent (Powell, 1993). Hence, in an effort to understand the effect of improved UV tolerance on the disease control efficacy under field conditions, a study was performed from July to October of 2009 and 2010. Various concentrations of Tx-1 or 10TC30, along with combinations with and without HA were tested for disease control. To further investigate the impact of HA on population size of the biocontrol agent in the field, populations in the foliage

and thatch of the treatments that were applied at 10^7 colony forming units (CFU) per cm^2 were monitored in 2010.

Materials and Methods

Field Condition

This study was conducted from July through October in 2009 and 2010 at the Hancock Turfgrass Research Center on the campus of Michigan State University, East Lansing, Michigan. The annual bluegrass (*Poa annua*) fairway was maintained at 1.27 cm. Fertility program was maintained as 113.4g of N/92.9m^2 biweekly during the study. Irrigation timing was adjusted to provide adequate moisture to maintain the annual bluegrass. Randomized complete block contained four replications was chosen as the statistical error control design. The plot sizes were set up as 1.37 by 0.61 m, with one foot buffer strips on two sides.

Bacterial Fermentation

Tx-1 or 10TC30 were grown for 48 to 72 hr before applying. 1 ml of frozen seed culture was transferred into 50 ml Tryptic soy broth (TSB) (Becton, Dickinson and Company. USA) and incubated for 24 hr. The 50 ml cultures were then inoculated to 1 L of TSB media. To achieve the optimal growth, the 1L cultures were incubated on a shaker (SK-71, Lab Companion, JEIOTECH. Korea) at 100 rpm with constant air circulation. Temperature was maintained at 25 ^{0}C.

Daily concentration of Tx-1 was measured by using a spectrophotometer. The absorbance values were translated into concentration of Tx-1 using a formula from a standard curve that was generated in previous studies (Powell, 1993).

Treatment application

The treatment list of 2009 and 2010 are shown in Table 2.01 and 2.02, respectively. Based on previous studies with Tx-1(Dwyer, 1999), two concentrations of Tx-1, 10^5 CFU/cm^2 and 10^7 CFU/cm^2, were chosen for this study. The concentration of HA (SIGMA-ALDRICH, Co. St. Louis, MO) was determined as 7.09 g/92.9m^2, which was calculated based on the results from *in vitro* study. Bacterial cultures and HA were mixed prior to each application. Banner Maxx (propiconazole), a chemical fungicide was applied biweekly as the positive control of the study. A nitrogen powered backpack sprayer was used to deliver all the treatments. The sprayer was calibrated to apply biological control agents at rate of 8.33 L/92.9m^2 and 4.16 L/92.9m^2 for Banner Maxx and HA. In 2009, biological treatments were delivered after 4:30 p.m. to achieve optimal condition for application and bacterial growth. This schedule was adjusted to 12:30 p.m. in 2010.

Table 2.01. List and application rates of treatments tested in 2009 field study for the suppression of dollar spot and anthracnose in East Lansing, Michigan.

Treatments	**Rate**	**Rate Unit**	**Application Interval**
P. aureofaciens Tx-1	2×10^5	CFU/cm^2	Five days a week
P. aureofaciens Tx-1	2×10^7	CFU/cm^2	Five days a week
P. aureofaciens Tx-1+ humic acid	2×10^5	CFU/cm^2	Five days a week
P. aureofaciens Tx-1+ humic acid	2×10^7	CFU/cm^2	Five days a week
humic acid	7.09	$g/92.9m^2$	Five days a week
Banner Maxx	0.03	$L/92.9m^2$	14 days
Untreated			

Table 2.02. List and application rates of treatments tested in 2010 field study for the suppression of dollar spot and anthracnose in East Lansing, Michigan.

Treatments	Rate	Rate Unit	Application Interval
P. aureofaciens Tx-1	2×10^5	CFU/cm^2	Daily
P. aureofaciens Tx-1	2×10^7	CFU/cm^2	Daily
P. aureofaciens Tx-1+ humic acid	2×10^5	CFU/cm^2	Daily
P. aureofaciens Tx-1+ humic acid	2×10^7	CFU/cm^2	Daily
10TC30	2×10^5	CFU/cm^2	Daily
10TC30	2×10^7	CFU/cm^2	Daily
10TC30 + humic acid	2×10^5	CFU/cm^2	Daily
10TC30 + humic acid	2×10^7	CFU/cm^2	Daily
humic acid	7.09	$g/92.9m^2$	Daily
Banner Maxx	0.03	$L/92.9m^2$	14 days
Untreated			

Field population of Tx-1

To measure the population sizes of bacterial treatments applied at 10^7 CFU/cm^2, rifampicin resistant strains of Tx-1 and 10TC30 were used. The procedure of selecting rifampicin resistant strains was described previously by Dwyer (1999). Three population samplings were arranged though out the study in 2010. The first sampling was collected at 14-Jul 2010, which was 24 hr after the first application. Second sampling was done at 27-Aug 2010, in the middle of the season. The third one was conducted at 10-Oct 2010, at the end of the field season. During every sampling date, three random samples were taken from each plot treated with 10^7 CFU/cm^2. All samples were taken using a soil probe (19.05 mm in diameter) that was surface-sterilized by 10% bleach and rinsed in dH_2O between each sample collection.

The collected samples were diluted and plated as previously described (Dwyer, 1999). In short, section from top 1 cm of the samples were dissected and transferred into 10 ml of PBS solution, pH 7.0. After serial dilution for each sample, 40 *ul* aliquots were plated on rifampicin amended media (50 mg/L) at various levels of dilutions in four replications. Colony counting was conducted following 48-72 hr of incubation period.

Data collection and analysis

Disease ratings and turf quality were taken every 7-14 days. The occurrence of disease was rated by visual estimation of the percent of each plot exhibiting disease symptoms. Turf quality was rated on a scale of 1-9, where 1=completely dead, 7= acceptable, and 9=excellent. A combination of features such as color, density, and uniformity were evaluated as the base of quality. Data was analyzed using ANOVA LSD (p=0.05) procedure from SAS (Statistical Analysis Software, Cary, N.C.).

Results

2009

In 2009, dollar spot ratings were taken on nine dates (Table 2.03). Tx-1, with and without HA, applied daily at the rate of 10^7 CFU/cm^2 provided significant control of dollar spot compared to the untreated control (Table 2.03). The level of disease reduction achieved by 10^7 CFU/cm^2 treatments was similar to the chemical fungicide Banner Maxx (Table 2.03). Significant dollar spot control was first observed at 29-Aug for Tx-1 10^7 CFU/cm^2 treatment, which is one rating date before Tx-1 HA treatment. Tx-1 alone applied at 10^5 CFU/cm^2 also provided significant dollar spot control since 29-Aug, but the level of disease suppression decreased followed by increased disease severity at 27-Sep and 14-Oct (Table 2.03). Tx-1+HA treatment applied at 10^5 CFU/cm^2 and HA alone treatments had no significant impact on dollar spot control (Table 2.03).

Table 2.03 Means and LSD comparisons for treatment effects on dollar spot disease incidence on different dates in 2009.

Treatments	Rate	Rate Unit	7/31	8/12	8/18	8/29	9/6	9/13	9/20	9/27	10/14
Tx-1	2×10^5	CFU/cm^2	0 a	0 a	2.8 a-c	2.9 bc	6.4 bc	11.3 b-d	8.8 bc	31.3 b	41.3 b
Tx-1	2×10^7	CFU/cm^2	0 a	0.5 a	4.5 a-c	2.5 bc	3 bc	6.3 d	3.5 c	10.5 c	10 c
Tx-1 + HA	2×10^5	CFU/cm^2	0 a	3 a	7.5 a-c	9.8 ab	13 ab	21.3 a-c	16.3 ab	45 ab	55 ab
Tx-1 + HA	2×10^7	CFU/cm^2	0 a	3.1 a	7 a-c	5.5 a-c	6.3 bc	7.5 cd	2.3 c	10.8 c	10.8 c
HA	7.09	$g/92.9m^2$	0 a	0.5 a	7 a-c	9.3 a-c	13.8 ab	25 ab	21.3 a	52.5 ab	60 a
Banner	0.03	$L/92.9m^2$	0 a	2.5 a	0.1 c	0.1 c	0 c	0.5 d	0 c	0 c	0 c
Untreated			0 a	1.9 a	10.8 a	12.5 a	19.5 a	31.3 a	23.8 a	57.5 a	63.8 a

Means followed by the same letter in a column are not significantly different according to Fisher's LSD ($P>0.05$).

Table 2.04 Means and LSD comparisons for treatment effects on anthracnose disease incidence on different dates in 2009.

Treatments	Rate	Rate Unit	7/31	8/12	8/18	8/29	9/6	9/13	9/20	9/27
Tx-1	2×10^5	CFU/cm^2	16.3 a	11.8 bc	16.3 bc	15.3 bc	21.3 a	8.8 ab	10.5 a	1.5 bc
Tx-1	2×10^7	CFU/cm^2	15 a	14.8 a-c	10 c	9.8 bc	7.5 bc	1.3 b	1.25 b	0 c
Tx-1 + HA	2×10^5	CFU/cm^2	18.8 a	23.8 a	31.3 a	17.5 a-c	18.8 ab	13 a	10.8 a	3.5 ab
Tx-1 + HA	2×10^7	CFU/cm^2	20 a	9.5 c	14.5 bc	5.5 c	5.75 c	2.5 b	1.3 b	0 c
HA	7.09	$g/92.9m^2$	20.5 a	13 a-c	30 a	30 a	20.5 a	10 ab	10 a	3.3 ab
Banner	0.03	$L/92.9m^2$	20 a	22.5 ab	13.8 bc	6.5 c	7 bc	9 ab	0 b	0 c
Untreated			15.5 a	11.3 bc	23.8 ab	22.5 ab	18 a-c	9.3 ab	11.3 a	4 a

Means followed by the same letter in a column are not significantly different according to Fisher's LSD ($P>0.05$).

Table 2.05 Means and LSD comparisons for treatment effects on turf quality on different dates in 2009.

Treatments	**Rate**	**Rate Unit**	7/31	8/12	8/18	8/29	9/6	9/13	9/20	9/27	10/14
Tx-1	2×10^5	CFU/cm^2	4.8 a-c	5 a	5 ab	4.8 bc	4.5 bc	4.5 bc	4.8 bc	4.3 bc	3.5 a
Tx-1	2×10^7	CFU/cm^2	4.8 a-c	4.8 ab	5 ab	5.3 ab	5.3 ab	5 b	5.3 ab	5.3 ab	3.5 a
Tx-1 + HA	2×10^5	CFU/cm^2	4.3 bc	4.5 ab	4.3 b	4.3 bc	3.8 c	3.8 c	4.3 b-d	3.8 c	3.3 a
Tx-1 + HA	2×10^7	CFU/cm^2	5 ab	4.5 ab	5.3 ab	5.3 ab	5.3 ab	5.3 ab	6 a	5.3 ab	3.5 a
HA	7.09	$g/92.9m^2$	4 c	4.3 b	4 b	3.8 c	3.8 c	3.8 c	4 cd	3.3 c	3 a
Banner	0.03	$L/92.9m^2$	5.3 a	4.5 ab	6 a	6.5 a	5.8 a	6.3 a	5 a-c	6 a	5 a
Untreated			4.8 a-c	4.5 ab	4 b	3.5 c	3.8 c	3.5 c	3.5 d	3.5 c	3 a

Means followed by the same letter in a column are not significantly different according to Fisher's LSD (P>0.05).

Anthracnose disease incidence in 2009 were taken and reported in Table 2.04. Significant suppression on anthracnose was achieved by 10^7 CFU/cm^2 treatments and Banner Maxx in 2009 at three rating dates (18-Aug, 20-Sep, 27-Sep for Tx-1; 29-Aug, 20-Sep, 27-Sep for Tx-1+HA and Banner Maxx) (Table 2.04). Tx-1 applied at 10^7 CFU/cm^2 was the first treatment that significantly controlled anthracnose on 18-Aug. No difference was observed among 10^5 CFU/cm^2 treatments, HA alone treatment, and the untreated control (Table 2.04).

Nine turf quality ratings were recorded in 2009 (Table 2.05). Compared to the untreated control, 10^7 CFU/cm^2 treatments showed significant improvement of turf quality from 29-Aug to 27-Sep (Table 2.05). The significant increase in turf quality for the chemical treatment (Banner Maxx) lasted from 18-Aug to 27-Sep, which was one rating date longer than the 10^7 CFU/cm^2 treatments (Table 2.05). Tx-1 applied at 10^5 CFU/cm^2 showed significant effect in turf quality at 20-Sep (Table 2.05). No difference in turf quality were observed among the Tx-1 + HA applied at 10^5 CFU/cm^2, HA alone treatment, and the untreated control (Table 2.05).

Table 2.06 Means and LSD comparisons for treatment effects on dollar spot disease incidence on different dates in 2010.

Treatments	Rate	Rate Unit	7/13	7/21	8/11	8/20	8/29	9/13	9/24	10/3
Tx-1	2×10^5	CFU/cm^2	3.4 a	12.8 a	10.3 a-d	14.3 ab	20.8 a	30 a	30 ab	25 a
Tx-1	2×10^7	CFU/cm^2	4.8 a	5.3 bc	7 b-e	5.8 c-e	9.3 bc	8.3 b	16.3 c-e	13.3 cd
Tx-1 + HA	2×10^5	CFU/cm^2	4.8 a	7.3 a-c	11.5 a-c	17.5 a	23.8 a	28.8 a	33.8 a	23.8 a
Tx-1 + HA	2×10^7	CFU/cm^2	5 a	4.3 bc	2.9 e	2 e	5.5 bc	8.5 b	13.8 de	11 d
10TC30	2×10^5	CFU/cm^2	5 a	10.8 ab	13.5 ab	14.3 ab	21.8 a	31.3 a	30 ab	21.3 ab
10TC30	2×10^7	CFU/cm^2	3.5 a	2.5 c	2 e	2.4 de	4.3 bc	8 bc	8.8 ef	11 d
10TC30 + HA	2×10^5	CFU/cm^2	4.8 a	9 a-c	8 b-e	9.3 b-d	23.8 a	27.5 a	22.5 b-d	19.3 a-c
10TC30 + HA	2×10^7	CFU/cm^2	6 a	8.3 a-c	4.5 c-e	7 c-e	11 b	9.8 b	13 de	15 b-d
HA	7.09	$g/92.9m^2$	5.3 a	10.8 ab	12 ab	11.5 a-c	20 a	28.8 a	26.3 a-c	21.3 ab
Banner	0.03	$L/92.9m^2$	3.5 a	2.8 c	3.5 de	5.8 c-e	1.6 c	0.3 c	0.1 f	1.3 e
Untreated			5.8 a	11.3 ab	16 a	16.3 ab	27.5 a	35 a	32.5 ab	22.5 ab

Means followed by the same letter in a column are not significantly different according to Fisher's LSD (P>0.05).

Table 2.07 Means and LSD comparisons for treatment effects on anthracnose disease incidence on different dates in 2010.

Treatments	**Rate**	**Rate Unit**	**7/21**	**8/2**	**8/11**	**8/20**	**8/29**
Tx-1	2×10^5	CFU/cm^2	21.3 a-c	16.3 a	9.3 bc	15 ab	8.8 a
Tx-1	2×10^7	CFU/cm^2	26.3 a-c	10.8 ab	6.3 c	6.8 c-e	5.5 a-c
Tx-1 + HA	2×10^5	CFU/cm^2	36.3 a	15 a	10.5 a-c	11.3 a-d	6.8 a-c
Tx-1 + HA	2×10^7	CFU/cm^2	14.5 bc	17.5 a	7.3 c	8.8 b-d	3.5 cd
10TC30	2×10^5	CFU/cm^2	18.8 a-c	19.3 a	11 a-c	13 a-c	4.3 b-d
10TC30	2×10^7	CFU/cm^2	27 ab	10.5 ab	7 c	6.3 de	4.3 b-d
10TC30 + HA	2×10^5	CFU/cm^2	17.5 a-c	16.8 a	10.5 a-c	10.5 a-d	7.5 ab
10TC30 + HA	2×10^7	CFU/cm^2	18.8 a-c	8.8 ab	5.5 cd	9.3 b-d	4 b-d
HA	7.09	$g/92.9m^2$	23.8 a-c	17.5 a	16.3 a	13.8 ab	5.8 a-c
Banner	0.03	$L/92.9m^2$	7.5 c	0 b	0.3 d	2.3 e	1.4 d
Untreated			23.8 a-c	19.5 a	14.3 ab	16.3 a	5.5 a-c

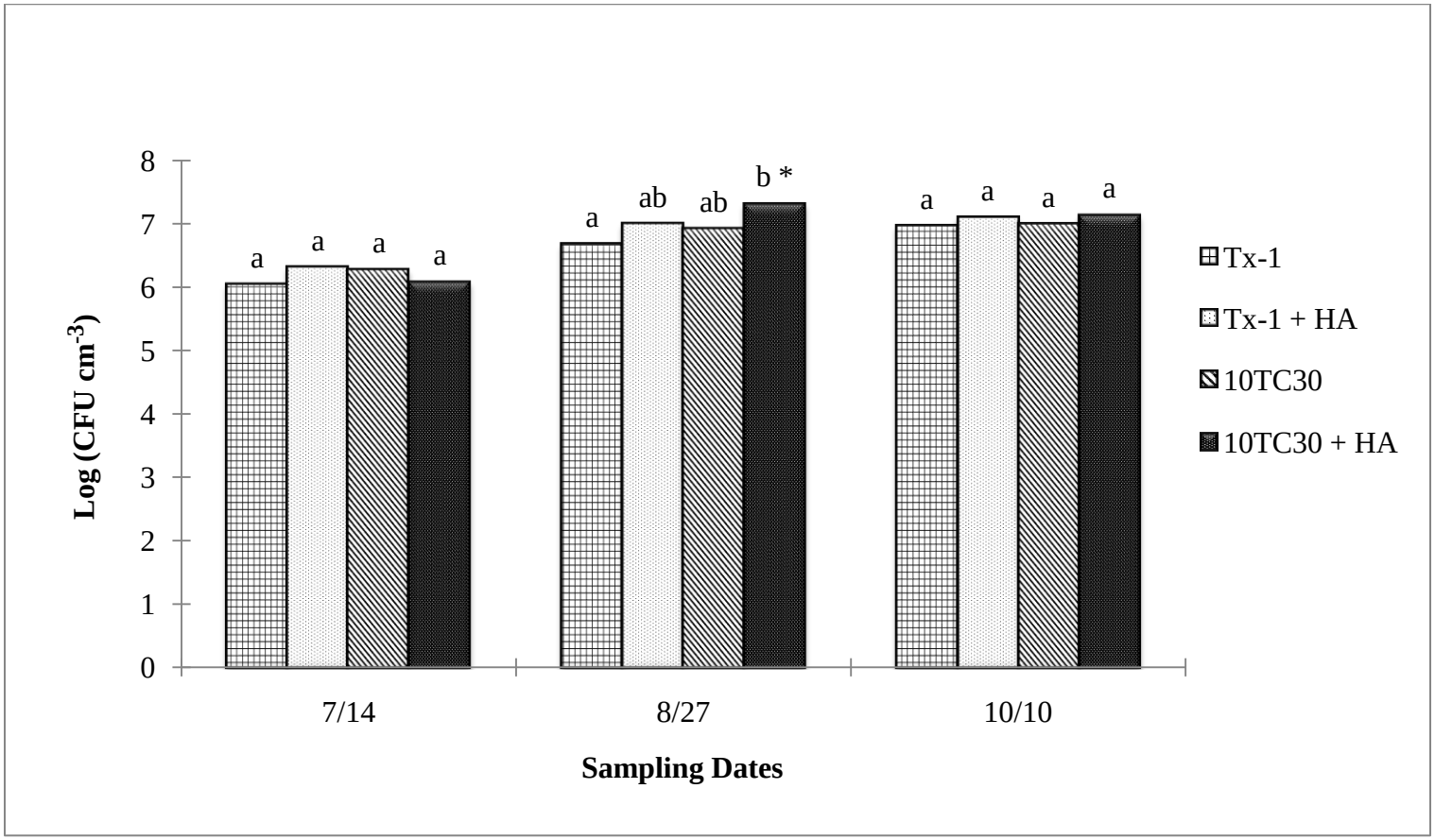
Means followed by the same letter in a column are not significantly different according to Fisher's LSD (P>0.05).

Table 2.08 Means and LSD comparisons for treatment effects on turf quality on different dates in 2010.

Treatments	Rate	Rate Unit	7/13	7/21	8/2	8/11	8/20	8/29	9/13	9/24	10/3
Tx-1	2×10^5	CFU/cm^2	5 a	4.5 b	4.8 d	4 f	4 c	3.8 de	3.8 c	3.5 ef	4 d
Tx-1	2×10^7	CFU/cm^2	4.8 a	4.8 b	5.5 b-d	5 c-e	5.5 b	4.8 cd	5 b	4.3 c-e	4.8 cd
Tx-1 + HA	2×10^5	CFU/cm^2	4.8 a	5 b	5.8 b-d	4.3 ef	3.8 c	3.5 e	3.5 c	3.3 f	4 d
Tx-1 + HA	2×10^7	CFU/cm^2	5 a	4.8 b	5.8 b-d	5.5 bc	5.5 b	5.3 bc	5 b	5 bc	5.3 c
10TC30	2×10^5	CFU/cm^2	4.5 a	4.5 b	5 cd	4.5 d-f	4.3 c	3.8 de	3.5 c	3.8 d-f	4.3 d
10TC30	2×10^7	CFU/cm^2	5 a	4.8 b	6.5 b	6.3 ab	5.5 b	6 ab	4.8 b	5.3 b	6.5 b
10TC30 + HA	2×10^5	CFU/cm^2	5 a	4.5 b	5.3 cd	4.5 d-f	4 c	3.8 de	4.3 bc	3.8 d-f	4.3 d
10TC30 + HA	2×10^7	CFU/cm^2	4.5 a	4.8 b	6 bc	5.3 cd	4.8 bc	4.8 cd	5 b	4.5 b-d	4.8 cd
HA	7.09	$g/92.9m^2$	4.8 a	4.5 b	5.5 b-d	4.8 c-f	3.8 c	4 de	3.8 c	3.8 d-f	4 d
Banner	0.03	$L/92.9m^2$	5 a	5.8 a	8.5 a	6.5 a	7 a	6.5 a	7 a	8.5 a	7.5 a
Untreated			4.5 a	4.8 b	5.5 b-d	4.8 c-f	4 c	3.5 e	3.8 c	3.3 f	4 d

Means followed by the same letter in a column are not significantly different according to Fisher's LSD (P>0.05).

Figure 2.01. Population size of *P. aureofaciens* Tx-1 in foliage and thatch.



*, Means followed by the same letter are not significantly different according to Fisher's LSD (P>0.05).

2010

In 2010, dollar spot disease incidence data that were collected from eight rating dates are presented in Table 2.06. 10TC30 applied at 10^7 CFU/cm^2 significantly suppressed the development of the disease on seven rating dates, which is the same duration of disease control provided by Banner Maxx (Table 2.06). The other three treatments applied at 10^7 CFU/cm^2 (Tx-1, Tx-1 + HA, and 10TC30 + HA) provided significant dollar spot control on six dates (11-Aug to 3-Oct). No differences were found among 10^5 CFU/cm^2 treatments with the only exception of 10TC30 + HA, which showed significant control of the disease on 11-Aug (Table 2.06).

The severity of anthracnose in 2010 was recorded at five rating dates is presented in Table 2.07. Significant disease control was achieved on two rating dates (11-Aug and 20-Aug) by biological treatments applied at 10^7 CFU/cm^2, which is two rating dates shorter than suppression provided by chemical fungicide (Table 2.06). All biological treatments applied at 10^5 CFU/cm^2 and HA alone treatment showed no effect on disease incidence (Table 2.07).

Turf quality ratings taken in 2010 at nine rating dates are reported in Table 2.08. Among all treatments applied at 10^7 CFU/cm^2, 10TC30 provided significantly improved turf quality on six rating dates, and the longest disease control period among all biological treatments (Table 2.08). Tx-1 and HA combination, Tx-1 alone, and 10TC30 + HA treatments applied at 10^7 CFU/cm^2 offered significant improvement on five, four, and three rating dates, respectively

(Table 2.08). 10^5 CFU/cm^2 treatments and HA alone treatment had no effect on turf quality (Table 2.08).

Populations of the top 1 cm soil (foliage and thatch) of the 10^7 CFU/cm^2 treatments were monitored in 2010 and presented in Figure 2.01. In 8/27, the 10TC30 + HA treatment had significantly higher log population size than Tx-1 alone treatment (Figure 2.01). No difference in the population was found among the treatments on any other rating dates (Figure 2.01).

Discussion

Humic acid has been shown as a promising UV sunscreen for Tx-1 *in vitro*. To investigate whether the improvement of UV tolerance provided by HA might affect disease control efficacy under field condition, Tx-1 with or without HA treatments were applied at 10^5 CFU/cm^2 and 10^7 CFU/cm^2 and tested over two years (2009-2010). When applied at 10^7 CFU/cm^2, Tx-1 and Tx-1 + HA treatments resulted in similar dollar spot suppression (Table 2.03, 2.06). Compares to Tx-1+HA applied at 10^7 CFU/cm^2, Tx-1 alone on one rating date provided significant control of dollar spot in 2009 (Table 2.03). Both treatments also provided significant control in 2010 (Table 2.06). This is the first study demonstrating that Tx-1 inhibits anthracnose *in vivo*. Results show Tx-1 and Tx-1 + HA applied at 10^7 CFU/cm^2 treatments provided similar levels of suppression of anthracnose (Table 2.04, 2.07). The similarity between Tx-1 and Tx-1 + HA treatments was observed in turf quality data as well, which show the same level of improvement on turf quality was achieved on similar rating dates in both years (Table 2.05, 2.08). Moreover, no significant difference of the population size of Tx-1 and Tx-1 + HA was observed

in 2010 (Figure 2.01). When applied at 10^5 CFU/cm^2, Tx-1 alone treatment provided significant dollar spot control at six rating dates in 2009, while Tx-1 + HA showed no effect on dollar spot incident (Table 2.03). No significant difference in disease incident and turf quality between Tx-1 and Tx-1 + HA was observed in 2010 (Table 2.06-2.08). HA alone treatment showed no effect on disease severity and turf quality for over two years (Table 2.03-2.08). All together, these results indicate that the addition of HA has limited effect on disease control efficacy of Tx-1 and HA itself has no impact on disease development.

One possible explanation of this is degradation of HA in the field. It has been shown that microbial population in aerobic environments are able to degrade humic materials (Trump, 2006). Exposure of HA to UV radiation from sunlight significantly promotes the bio-degradation process (Trump, 2006). Since it has been proposed that the UV inactivation efficacy of HA could be concentration dependent (Cantwell, 2008), it is likely that the bio-degradation of HA caused by microbes in the field might decrease the concentration to a level that it is unable to provide efficient UV protection for Tx-1. To understand more about the degradation process of HA, concentrations of HA in the field need to be traced and investigated in future field studies.

The competition with the other micro-flora is crucial for an introduced biocontrol agent to survive and colonize (Thomashow, 1988). The survival and colonization of bacteria or microbial biocontrol agents in UV intensive habitats, such as the leaf surface, largely depend on tolerance to radiation (Sundin, 2004). HA has been shown to be able to protect numerous species of bacteria from UV radiation (Corin, 1998; Templeton, 2006; Cantwell, 2008; Lee, 2009). It is reasonable to hypothesize that the application of HA might not only protect Tx-1, but other microbes as well. This improved UV tolerance provided by HA to the other microbes on the leaf

would benefit their survival hence increasing the competition in the area. This might be a possible explanation for the observation that in 2009, when all the treatments were applied after 4:30 pm., Tx-1 + HA at 10^5 CFU/cm^2 failed to inhibit dollar spot (Table 2.04), and at 10^7 CFU/cm^2 rate, Tx-1 alone provided on one rating date more control of dollar spot. Moreover, after the application schedule was changed to noon in 2010, the overall solar UVR was more intensive than 2009, during which HA could be degraded faster, this differences in disease control efficacy among Tx-1 alone treatments and Tx-1 + HA treatments at both application rates were not been observed (Table 2.06, 2.07). To fully understand the impact of HA on other microbes on the leaf, population size of these microbes needed to be traced in further studies.

The disease suppression efficacy of 10TC30 with and without HA was evaluated in the field in 2010. 10TC30 applied at 10^7 CFU/cm^2 was the first biological treatment that significantly controlled dollar spot on 21-Jul (Table 2.06). It also provided longer period of improved turf quality than Tx-1 treatments (Table 2.08). 10TC30 has shown improved UVC/UVB tolerance *in vitro* (Figure 1.06). This increased UV-B tolerance might provide 10TC30 an advantage in colonizing the area faster than Tx-1, which could be a likely explanation for this longer period of dollar spot control. 10TC30 + HA provided similar dollar spot and anthracnose control as Tx-1 treatments (Table 2.06, 2.07). The addition of HA to 10TC30 at 10^7 CFU/cm^2 rate failed to provide control for dollar spot on 21-Jul (Table 2.06). It might be a result of HA protecting other microbes and increasing the competition in that area.

Population sizes for treatments applied at 10^7 CFU/cm^2 were monitored in 2010. The population sizes of all the 10^7 CFU/cm^2 treatments were not different from each other after 24 h

of first application (Figure 2.01), and the value were all one log factor lower than 10^7 which been applied. This observation is in the agreement with previous field study with Tx-1, which reported the threshold population of disease control was one log factor lower than the daily application rate (Dwyer, 1999).The population size of 10TC30 + HA at 27-Aug was significantly larger than Tx-1 alone treatment (Figure 2.01). The increased UV-B tolerance of 10TC30 and UV protection provided by HA might be the reasons which lead to the increase population size. It could also be a possible explanation that when applied at 10^5 CFU/cm^2, 10TC30 + HA was the only treatment that inhibited dollar spot on one rating date (8/11) (Table 2.06). The increased population size for 10TC30 + HA treatment was not observed at the last sampling date (Figure 2.01), it is possible this was due to the shorter days which decreased the intensity of UV radiation and the temperature. UV-A wavelengths attribute 95% of total energy within solar UVR (Sundin, 2004), 10TC30 has shown no change in UV-A tolerance *in vitro* (Table 1.06), this could be a possible reason for the fact that population of 10TC30 alone treatment was no different to Tx-1.

It has been suggested that the mechanism for Tx-1 to colonize and compete in the canopy is antibiosis-asisted competition (Sigler, 2001). Based on data reported by Powell (1993), the antibiotic of Tx-1 was secreted primarily during the stationary phase, during which the population size of Tx-1 was around 9 to 9.5 log of CFU. Hence, it has been proposed that the antibiotics produced by Tx-1 during the incubation period may be the major factor in fungal inhibition (Dwyer, 1999; Sigler, 2001). Data reported by Powell (1993) and Dwyer (1999) support this hypothesis by showing that the heat-killed Tx-1, and purified antibiotic of Tx-1 (PCA) were sufficient for disease control. As a result, it is reasonable to hypothesize that the

increased population size of 10TC30 + HA might not be large enough to promote the production of the antibiotics. It is a possible explanation for the observation that the advantage in population size of 10TC30 +HA treatment did not result in an improvement in disease suppression efficacy (Table 2.06, 2.07). To further understand the disease control efficacy of Tx-1 in the field, the relationship between the population of Tx-1 and its antibiotic production need to be investigated *in vivo*.

In most cases, biological control agents are delivered to an ecological unsuitable environment (Deacon, 1991). Besides UV inhibition, the survival and biological antagonistic activity of biocontrol agents are highly influenced by other environmental conditions such as humidity, pH, nutritional availability, temperature (Lahlali et al., 2011). Hence, the improvement in only UV tolerance might not be conclusive for increasing the overall persistent and disease control efficacy of Tx-1. In order to fully optimize the disease control efficacy of Tx-1, the impacts from other environmental factors need to be studied.

LITERATURE CITED

Baker, K.F. (1987). Evoloving concepts of biological control of plant pathogens. Annual Review of Phytopathology **25,** 67-85.

Bull, D.L.R., R. L.; House, V. S.; Pryor, N. W. (1976). Improved formulations of the Heliothis nuclear polyhedrosis virus. Journal of Economic Entomology **69,** 731-736.

Cantwell, R., Hofmann, R. and Templeton, M. (2008). Interactions between humic matter and bacteria when disinfecting water with UV light. Journal of Applied Microbiology **105,** 25-35.

Chang, C.J., Floss, H. G., Hook, J.A., Mabe, J.A., . (1981). The Biosynthesis of the Antibiotic Pyrrolnitrin by *Pseudomonas aureofaciens*. The Journal of Antibiotics. **34,** 555-566.

Cook, R.J. (1993). Making greater use of introduced microorganisms for biological control of plant pathogens. Annual Review of Phytopathology **31,** 53-80.

Cooper, R.J., Liu, C., Fisher, D.S. (1998). Influence of Humic Substances on Rooting and Nutrient Content of Creeping Bentgrass. Crop Science **38,** 1639.

Corin, N., Backlund, P., Wiklund, T. (1998). BACTERIAL GROWTH IN HUMIC WATERS EXPOSED TO UV-RADIATION AND SIMULATED SUNLIGHT. Chemosphere**,** 1947-1958.

Crouch, J.A., E. N. Weibel, J. C. Inguagiato, et al. . (2003). Suppression of anthracnose on an annual bluegrass putting green with selected fungicides, nitrogen, plant growth regulators, and herbicides. 2003 Rutgers Turfgrass Proceedings **35,** 183-192.

Deacon, J.W. (1991). Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens. Biocontrol Science and Technology **1,** 5-20.

Dwyer, P.J., Jr., . (1999). Field Efficacy, Persistence, and Antibiotic Production of *Pseudomonas auerofaciens.* In Department of Botany and Plant Pathology (East Lansing: Michigan State University).

Dyke, A.V., Johnson, P.G., Grossl, P.R. (2009). Influence of humic acid on water retention and nutrient acquisition in simulated golf putting greens. Soil Use and Management **25,** 255-261.

Eisenstark, A. (1987). Mutagenic and lethal effects of near-ultraviolet radiation (290-400 nm) on bacteria and phage. Environmental and Molecular Mutagenesis **10,** 317-337.

Garcia-Pichel, F. (1994). A model for internal self-shading in planktonic organisms and its implications for the usefulness of ultraviolet sunscreen. Limnol Oceanogr **39,** 1704-1717.

Gomes, A.I., Santos, J.C., Vilar, V.J.P., Boaventura, R.A.R. (2008). Inactivation of Bacteria *E. coli* and photodegradation of humic acids using natural sunlight. Applied Catalysis B: Environmental **88,** 283-291.

Griffiths, H.R., Mistry, P., Herbert, K.E., Lunec, J. (1998). Molecular and cellular effects of ultraviolet light-induced genotoxicity. Clinical Laboratory Sciences **35,** 189-237.

Hadapad, A.B., Hire, R.S., Vijayalakshmi, N., Dongre, T.K. (2009). UV protectants for the biopesticide based on Bacillus sphaericus Neide and their role in protecting the binary toxins from UV radiation. Journal of Invertebrate Pathology **100,** 147-152.

Hardebeck, G.A., R. F. Turco, R. Latin, and Z. J. Reicher. (2004). Application of Pseudomonas aureofaciens Tx-1 through irrigation for control of dollar spot and brown patch on fairway height turf. HortScience **39(7),** 1750-1753.

Harper, D.R. (2006). Biological contorl by microoorganisms. In Encyclopedia of Life Sciences (John Wiley & Sons.

Jacobs, J.L., Sundin, G.W. (2001). Effect of Solar UV-B Radiation on a Phyllosphere Bacterial Community. Applied and Environmental Microbiology **67,** 5488-5496.

Jacobs, J.L., Carroll, T.L., Sundin, G.W. (2004). The Role of Pigmentation, Ultraviolet Radiation Tolerance, and Leaf Colonization Strategies in the Epiphytic Survival of Phyllosphere Bacteria. Microbial Ecology **49,** 104-113.

Kim, J.J., Sundin, G.W. (2001). Construction and Analysis of Photolyase Mutants of *Pseudomonas aeruginosa* and *Pseudomonas syringae*: Contribution of Photoreactivation, Nucleotide Excision Repair, and Mutagenic DNA Repair to UV-B Radiation. Applied and Environmental Microbiology **67,** 1405-1411.

Kluver, A.J. (1956). Pseudomonas aureofaciens Nov. Spec. and its Pigments. The Journal of Bacteriology **72,** 406-411.

Lahlali, R., Raffaele, B., and Jijakli, M.H. (2011). UV protectants for Candida oleophila (strain O), a biocontrol agent of postharvest fruit diseases. Plant Pathology **60,** 288-295.

Lee, E., Lee, H., Jung, W., Park, S., Yang, D., Lee, K. (2009). Influences of humic acids and photoreactivation on the disinfection of Escherichia coli by a high-power pulsed UV irradiation. Korean Journal of Chemical Engineering **26,** 1301-1307.

Murphy, J., F. Wong, L. Tredway, et al. (2008). Best management practices for anthracnose on annual bluegrass turf. Golf Course Management **76(8),** 93-104.

Nikolai, T.A., Rieke, P.E., Rogers, J.N. III, and Vargas, J.M.Jr. . (2001). Thrfgrass and soil responses to lightweight rolling on putting green root zone mixes. . International Turfgrass Society Research Journal **9,** 604-609.

Palleroni, N.J. (1984). Genus I. Pseudomonas Migula 1894. In Bergey's manual of systematic bacteriology, H.J.G. Krieg N R, ed (Baltimore: The Williams & Wilkins Co.), pp. 141-199.

Pfeifer, G.P. (1997). Formation and processing of UV photoproducts: effects of DNA sequence and chromatin environment. Photochem. Photochemistry and Photobiology **65,** 270-283.

Pierson III, L.S.a.P., E. A. (1996). Phenazine antibiotic production in Pseudomonas aureofaciens: role in rhizosphere ecology and pathogen suppression. FEMS Microbiology Letter **136,** 101-108.

Pierson, L.S.a.L.S.T. (1992). Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30-84. Molecular Plant-Microbe Interactions **5,** 330-339.

Powell, J.F. (1993). Utilization of Bacterial Metabolites for the Management of Fungal Turfgrass Pathogens. In Department of Botany and Plant Pathology (East Lansing: Michigan State University).

Powell, J.F., Vargas, J. M., Jr., et al. (2000). Management of dollar spot on creeping bentgrass with metabolites of Pseudomonas aureofaciens (TX-1). Plant Disease **84,** 19-24.

Pusztai M, F.P., Cringorten L, Kaplan H, Lessard T, Carey PR. (1991). The mechanism of sunlight-mediated inactivation of Bacillus thuringiensis crystals. Biochemical Journal **273,** 43-47.

Putman, A.I., Jung, G., and Kaminski, J. E. (2010). Geographic distribution of fungicideinsensitive Sclerotinia homoeocarpa isolates from golf courses in the northeastern United States. Plant Disease **94,** 186-195.

Sigler, W.V., Nakatsu, C. H., Reicher, Z. J., Turco, R. F. (2001). Fate of the Biological Control Agent Pseudomonas aureofaciens Tx-1 after Application to Turfgrass. Applied and Environmental Microbiology **67,** 3542-3548.

Smiley, R.W., Dernoeden, P. H., and Clarke, B. B. (2005). Compendium of Turfgrass Diseases. (American Phytopathological Society. St. Paul, MN).

Stack, J.P., Kenerley, C. M., and Pettit, R. E. (1988). Biocontrol of Plant Disease. In Application of biological control agents, e. K. G. Mukerju and K. L. Grag, ed (CRC Press, Boca Raton, FL.), pp. 43-54.

Steinberg, C.E.W. (2004). Ecology of Humic Substances in Freshwaters. (Verlag Berlin Heidelberg: Springer).

Sundin, G.W., Jacobs, J.L. (1999). Ultraviolet Radiation (UVR) Sensitivity Analysis and UVR Survival Strategies of a Bacterial Community from the Phyllosphere of Field-Grown Peanut (Arachis hypogeae L.). Microbial Ecology **38,** 27-38.

Sundin, G.W., Kidambi, S.K., Ullrich, M., Bender, C.L. (1996). Resistance to ultraviolet light in *Pseudomonas syringae*: sequence and functional analysis of the plasmid-encoded *rulAB* genes. . Gene **177,** 77-81.

Tamez-Guerra, P., Rodriguez-Padilla, C., and Galán-Wong Luis, J. (2005). Solar Radiation (UV) Protectants for Microbial Insecticides. In Semiochemicals in Pest and Weed Control (American Chemical Society), pp. 127-147.

Templeton, M.R., Hofmann, R., Andrews, R.C. (2006). UV inactivation of humic-coated bacteriohages MS2 and T4 in water. Environmental Engineering Science **5,** 537-543.

Thomashow, L.S., and Weller, D. M.,. (1988). Role of a Phenazine Antibiotic from *Pseudomonas fluorescens* in Biological Control of *Gaeumannomyces graminis var. tritici.* . The Journal of Bacteriology **170,** 3499-3508.

Thomashow, L.S., Weller. D. M., Bonsall, R. F. and Pierson. L. S. III. (1990). Production of a phenazine antibiotic by fluorescent Pseudomonas species in the rhizosphere of wheat. Applied and Environmental Microbiology **56,** 908-912.

Towers, G., K. Green, E. Weibel, P. Majumdar and B. B. Clarke. (2003). Evaluation of fungicides for the control of anthracnose basal rot on annual bluegrass. Fungicide and Nematicide Tests **58,** T017.

Trump, J.I.V., Sun, Y., Coates, J.D. (2006). Microbial Interactions with Humic Substances. Advances in Applied Microbiology **60,** 55-96.

Turner, J.M.a.M.A.J. (1986). Occurrence, biochemistry and physiology of phenazine pigment production. Advances in Microbial Physiology **27,** 211-275.

Uyguner, C.S., and Bekbolet, M. (2005). Evaluation of humic acid photocatalytic degradation by UV-vis and fluorescence spectroscopy. Catalysis Today **101,** 267-274.

Vargas, J.M.J. (2005). Management of turfgrass diseases. . (Hoboken, New Jersey: John Wiley & Sons, Inc.).

Wang, Y., Casadavell, A. (1994). Decreased susceptibility of melanized *Cryptococcus neoformans* to UV light. Applied and Environmental Microbiology **60,** 3864-3866.

Weigand, M.R., Sundin, G.W.,. (2009). Long-term effects of inducible mutagenic DNA repair on relative fitness and phenotypic diversification in *Pseudomonas cichorii* 302959. Genetics **181,** 199-208.

Weigand, M.R., Tran, V.N., Sundin, G.W.,. (2011). Growth Paraeter Components of Adaptive Specificity during Experimental Evolution of the UVR-Inducible Mutator *Pseudomonas cichorii* 302959. PUBLIC LIBRARY of SCIENCE One **6,** e15975.

Weller, D.M. (2007). Pseudomonas biocontrol agents of soilborne pathogents: Looking back over 30 years. Phytopathology**,** 250-256.

Wong, F.P., and S. Midland. (2004). Fungicide resistant anthracnose: bad news for greens management. Golf Course Management **72(6),** 75-80.

Wood, D.W., Pierson III, L.S.,. (1996). The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. Gene **168,** 49-53.