Effects of Microbial Inoculants on Biocontrol and Plant Growth Promotion

THESIS

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By

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Abstract

The expansion of organic agriculture has increased the demand for alternatives to chemical fertilizers and pesticides. Biopesticides and biofertilizers are part of a set of natural-based products being widely use to enrich the quality of the soil and control pests. In this study, a series of greenhouse and field bioassays were carried out with the aim of determining the biocontrol and biofertilizer effects of treatments prepared with *Mitsuaria* sp. strain H24L5A and *Burkholderia* sp. strain R4F2 in comparison with a commercially-available *Streptomycyes* inoculant on plants. Additionally, the effects of an experimental algae-based biofertilizer and commercial and pre-commercial biofertilizer products were assessed. First, a bioassay method was developed to successfully detect plant growth promotion and reduction of disease severity when the different treatments were applied by drenching. At a concentration of $10^7$ cells/ml, *Mitsuaria* sp. provided the greatest biocontrol of bacterial leaf spot ($P < 0.05$) with a 39% reduction as compared to the negative control in tomato greenhouse bioassays. Although not statistically significant, field experiments showed a trend also indicating a 12% reduction in bacterial leaf spot by *Mitsuaria* sp.. *Mitsuaria* sp. and *Burkholderia* sp. treatments provided the greatest enhancements of shoot growth and biomass ($P < 0.05$) up to 64% as compared to the negative controls in tomato greenhouse bioassays. These data indicate the biocontrol and plant growth promoting activities of the experimental bacterial ingredients. Furthermore, the results indicated that these unformulated inoculants have utility comparable to that of
commercial formulations. This is the first report of *Mitsuria* sp. strain H24L5A and *Burkholderia* sp. strain R4F2 as biocontrol agents of bacterial leaf spot and plant growth promotion agents of tomato and wheat.
Dedication

To my beloved family, the core of who I am:

Graciela Miranda, my mom, my inspiration, my strength, my shelter and support,

Alfonso Cepeda, my dad, my inspiration, my support, my example of humility, my angel in heaven,

Jorge Cepeda, my brother, my friend, my reference, my strength, my support
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CHAPTER 1

Introduction

Plant-associated microorganisms as beneficial actors for plant’s health and growth promotion

Plants can be the richest habitats for microbial growth. Their different zones, especially those full of moist and nutrients, their surfaces and tissues can be suitable for the proliferation of microorganisms (Andrews, 2000; Beattie, 2006). In fact, depending on their behavior, these microorganisms can be beneficial or pathogenic, or even neutral. The main focus of this study is the beneficial group of microorganisms that can serve as biofertilizers or biopesticides.

Due to the different composition of its structures, plants offer a wide range of different habitats suitable for microorganism’s growth. Microorganisms can inhabit the spermosphere, a zone influenced by the seeds, which is full of nutrients that support their growth. In addition, they can inhabit the phyllosphere, a zone that comprises the above ground parts of the plants, whose most relevant characteristic is the fact that is in constant fluctuation related to external facts as temperature, radiation and water availability. Other zones correspond to the vascular tissue, the rhizosphere and the endophytic sites.
The vascular tissue, composed by xylem and phloem, constitutes a set of rich environments for bacterial growth. In addition, endophytic bacteria include that colonize healthy plant tissue without causing injury, are important ecologically due to this fact (Beattie, 2006).

Moreover, is in the rhizosphere, region that includes plant roots and surrounding soil, where intensive interactions between plants, soil and microfauna take place due to its high energy and C content. This accumulation in the rhizosphere corresponds to all the compounds produced by plant roots, most of which are organic derived from photosynthesis and other plant processes (Pinton et al., 2001). Different and varied biochemical signal exchanges take place between these communities and their host plants; indeed, a wide diversity of bacteria and plant-associated microbes can interact with plants in a beneficial way either by enhancing their growth and/or controlling diseases (Beattie, 2006; Nihorimbere et al., 2011). Indeed, related to their enhancing activity, it is known that the majority of bacteria that promote plant growth are rhizosphere inhabitants; they have been designated as plant growth-promoting rhizobacteria, or PGPR. In fact, there are some products based on PGPR that have been used for agriculture due to their plant growth promoting potential and also for their biocontrol potential. According to Harman et al., (2010), some PGPR products that can act as biocontrol are not registered as biopesticides due to specific regulations and requirements that take more time to be fulfilled; instead, they are commercialized as plant inoculants.

In general, microorganisms related to plants include prokaryotic and eukaryotic domains. The bacterial domain is the most dominant component in the microflora,
achieving densities of approximately $10^9$ cells per gram of plant tissue of roots. On the other hand, the eukaryotic domain which includes filamentous fungi, yeasts, algae, protozoa and nematodes, can only be present in lower densities like $10^5$-$10^6$ fungi, $10^3$ algae and $10^2$-$10^3$ protozoa cells per gram of root (Beattie, 2006). In addition, the presence of viruses and bacteriophages has also been reported on soil with a density of $10^8$ to $10^9$ cells per gram of soil (Ashelford, 2003).

Three different categories have been established to describe the beneficial microbial interactions with plants. The first category corresponds to microorganisms responsible for the plant’s nutrition, which may interact direct or indirectly with the plant, like nitrogen fixation bacteria. The second to microorganisms responsible for stimulating plant growth indirectly, this means by preventing the growth of pathogens. In addition, the microorganisms belonging to this category are known as biocontrol agents. The third category involves the microorganisms that act directly into the plant’s growth by the production of phytohormones, solubilization of phosphates, the increase of iron uptake, and production of volatiles (Nihorimbere, 2011; Podile and Kishore, 2006). Many plant-associated microbes, even the ones that are in less proportion can have capacities that can be of great help from the agriculture and environmental point of view (Beattie, 2006).

*Use of microbial inoculants as biopesticides and biofertilizers*

As a response to the extended use of pesticides and the abuse of chemical compounds on fertilizers and pesticides all over the world, the use of biofertilizers and bacterial inoculants as biocontrol agents has gained especial relevance in agriculture. In fact, there is great potential on the use of biopesticides and biofertilizers in organic and
conventional agriculture (McSpadden Gardener and Fravel, 2002). Moreover, according to Berg (2009) there is a growing market for microbial inoculants across the world that has an annual rate of approximately 10%. Other important facts of microbial inoculants when compared with chemical pesticides and fertilizers are: a) they are more safe, b) show reduced environmental damage, c) show more targeted activity, d) are effective in smaller quantities, e) are able to multiply but are also controlled by the plant and indigenous microbes, f) have quicker decomposition procedures, g) are less likely to induce resistance by the pathogens and pests and finally g) can be used either in organic and conventional agriculture (Berg, 2009).

Plant growth and disease suppression are the most important facts that confer liability to products used in agriculture. The nature of these products may vary; typically they can be derived from natural materials (animal, plants, bacteria and certain minerals) or have a chemical basis. Accordingly, “plant strengthening agents”, “inoculants”, “biofertilizers” and “plant growth enhancer microbially-based products”, are terms applied to organisms sold to improve plant performance (Harman, 2010). In addition, the term biofertilizer is a recently coined term that commonly refers to the use of soil microorganisms to increase the availability and uptake of mineral nutrients for plants. Their mode of action is the stimulation of plant growth by controlling pathogenic organisms that can cause disease (Vessey, 2003). On the other hand, chemical based agricultural fertilizers are based in the production of nitrogen, phosphorous and potassium products, which are the most important nutrients for plant growth. In the case of pesticides, the nature can differ as well, but the most important distinction among the
natural derived ones and the chemical based is the potential harm they can cause: biopesticides pose little or no risk to human health or the environment (EPA, 2000).

Diversity of microbes used as inoculants

While different interactions have been described, different microorganisms and their combinations have been the focus of study of Agricultural Biotechnology as commercial products known as biofertilizers and biopesticides. One group of them corresponds to plant growth-promoting rhizobacteria (PGPR) that are rhizosphere non-pathogenic microorganisms that improve plant’s growth. PGPR promote plant growth by improving the absorption of minerals and water and the production of plant growth-stimulating compounds. In addition, growth’s improvement can happen due to the suppression of harmful microorganisms (de Weert and Bloemberg, 2006). Another group of described microorganisms is the *Rhizobium* genre, a group of gram-negative bacteria responsible of fixing nitrogen by the establishment of symbiosis with leguminous plants; *Rhizobium* has been used as a basis of inoculants that have been used as biofertilizers (Sessitsch et al., 2002).

Plant growth promoting rhizobacteria induce plant growth either direct or directly. Direct mechanisms comprise the production of substances like phytohormones, liberation of nutrients and stimulation of induced systemic resistance. For example, PGPR can produce IAA (indole-3-acetic acid), an auxin that contribute to plant growth promotion, generally described in *Azospirillum brasilense*, *Agrobacterium* spp., *Bradyrihizobium* spp., *Enterobacter* spp., and *Rhizobium leguminosarum*. On the other hand, indirect mechanisms comprise stimulation of symbiotic relationships, root growth
stimulation and biocontrol (Siddiqui, 2006; Vessey, 2003). For example, genera like *Azospirillum, Bacillus* and *Pseudomonas* can enhance legume symbioses (Podile and Kishore, 2006). Moreover, it is important to know that in some cases, several mechanisms are involved when it comes to beneficial plant microbe interactions (Nihorimbere et al., 2011). Thus, the identification of the mechanisms responsible of plant growth represents a big challenge due to the variety of them and the difficulty in measuring growth under different biotic or abiotic conditions (Podile and Kishore, 2006).

In general, PGPR comprise a group of genera that includes *Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Klebsiella* and *Pseudomonas* and some endophytes that include *Azoarcus* spp, *Gluconacetobacter diazotrophicus* and *Herbaspirillum seropedicae* (Siddiqui, 2006). *Azospirillum*, genera isolated of grasses and cereals, fixes atmospheric nitrogen as well as *Azoarcus* sp., *Burkholderia* sp., *Gluconacetobacter diazotrophicus, Herbaspirillum* sp., *Azotobacter* and *Paenibacillus polymyxa* (Vessey, 2003). In addition, *Bacillus* spp. species have been reported to promote plant growth and biological control of microbial diseases in a wide range of plants due to their capacity of inhibitory volatile substances. In addition, Pseudomonads have shown beneficial effects on plant yield and biological control, especially of *Fusarium* wilts. Moreover, Rhizobia group are known as “microbial symbiotic partners” that can produce phytohormones and other compounds that stimulate plant growth and have shown positive effects as biocontrol agents for *Pythium* diseases (Antoun and Prévost, 2005).

Several genera of PGPR and other microorganisms have been used in commercial products after having shown positive results either as biofertilizers or
biological control agents; indeed, the first commercial preparations appeared in 1990 (Bashan et al., 2004). According to Lucy et al (2004), *Azospirillum* species heads a long list of commercial free living PGPR products that are applied to crops in formulations. Some of them protect plants as biocontrol agents and some promote plant growth as well. Additionally, among the most important species of PGPR used for commercial products is *Bacillus subtilis* under the trade names Serenade, Kodiak, etc. The target crops are beans, cotton, legumes, pea, rice and soybean. Moreover, another well known species used as the basis of a commercial product is *Agrobacterium radiobacter*, under the trade names Diegall, Nogall, etc. In this case, the target crops are: fruit, nuts, ornamentals and trees. Finally, *Pseudomonas fluorescens* has been used to produce commercial inoculants under the trade names Conquer and Victus (Lucy et al., 2004).

*Azospirillum* is a genus of free-living PGPR very important in the areas of agriculture and ecological sciences and one of the best characterized (Bashan et al., 2004; Somers and Srinivasan, 2004). Among the mechanisms described as responsible for growth promotion by *Azospirillum* are 1) production of phytohormones (auxin indole-3-acetic acid is the most important phytohormones produced by *Azospirillum*), 2) atmospheric nitrogen fixation and 3) nitrate reduction. Moreover, greenhouse and field experiments have revealed increases in the absorption of minerals and increases in root elongation in inoculated plants (Somers and Srinivasan, 2004). *Azospirillum* can associate with *Pseudomonas* in order to colonize rapidly the root of the plants, which represents a clue about the combination of two different genera that can work together as bacterial inoculants (Somers and Srinivasan, 2004).
Bacillus is a genus of bacteria known to elicit induced systemic resistance (ISR) in plants. In addition, Bacillus spp. have reduced incidence of viral diseases, for instance, Cucumber Mosaic Virus on tomato. On plants that are not challenged with pathogens, it has been reported that Bacillus can increase fresh weight and number of fruits and flowers (Kloepper et al., 2004).

Pseudomonas, a genus of bacteria that can colonize plant roots and suppress pathogens through the production of antibiotics, is a genus that can elicit ISR as well (Kloepper et al., 2004). Bacteria in this genus have a strong potential as biocontrol and growth-promoting agents due to the following characteristics a) rapid growth in vitro, b) rapid utilization of seed and root exudates, c) ability to colonize and multiply in the rhizosphere and the spermosphere as well as inside the plants, d) production of metabolites like antibiotics, siderophores and growth promoters, e) competition with other microorganisms, and finally f) ability to adapt to environmental stress (Weller, 2007). In early attempts, products made of this Bacillus spp. failed due to the instability of the culture and lack of long term viability (Kloepper et al., 2004).

Another group of microbes corresponds to some types of algae that have biotechnological potential as soil fertilizers for plant production, and some macroscopic marine algae (Eklonia maxima) that improve the growth and yield of plants (Reisser, 2010; Crouch and van Staden, 1993). Finally, several genera and species of PGPR and different microbes are used as inoculants; the diversity represents an opportunity to start research in this area and provide new solutions for the current necessities of agriculture.
Formulations used for inoculation of beneficial microorganisms

A formulated microbial product is a product composed of one or more biological control agents mixed with ingredients that will improve its survival and effectiveness (Schisler et al., 2004).

Among the variety of inoculants, the ones formulated for delivery to soil are of especial importance due to their specific court of action which is the rhizosphere. Specifically, bacterial inoculants can be applied to the soil as fluid suspensions, powder formulations and as granules for soil and spray application. Fluid suspensions are prepared based on culture concentrates diluted in water or a buffer solution prior application. They can also be prepared as dormant aqueous suspensions, obtained after harvesting the bacteria from a liquid culture, washed free of the spent medium and stored at an specific concentration in sterile water at room temperature. *Rhizobium* products have been prepared this way and applied as sprays by dilution in water. Powder formulations consist of organisms concentrated into dry or wet powders. Depending on the composition of the powders, they can be applied directly to the soil, suspended in water or dusted onto seeds. The commonest method to formulate granular products is to mix the organism and the ingredients with the granules (Burges, 1998).

Inoculants formulations depend directly on the carrier used for the delivery of the products because target microorganisms are added to it before being applied. Carriers are the inert ingredients that hold or dilute the microorganism to the desired concentration and improve coverage and distribution (Burges, 1998). Carriers constitute the key for the effective release of the different products, they need to be effectively chosen due to their diversity (e.g. water, vermiculite, calcium sulphate, mineral soil and sand, vegetable oil,
corn cob) that stats form classic ones to new and unconventional ones (Bashan, 1998; Burges, 1998).

The carrier represents the principal portion of inoculants. The materials from which they are made define their effectiveness. Moreover, they have to fill certain requirements in order to be efficient. First, they need to have the capacity to deliver the correct concentration of viable cells at the time they are needed. The reason is because there are certain ranges of concentrations that can be inoculated in certain crops. In addition, as inoculants should be sterile, carriers should be chemically consistent and able to provide enough water holding capacity for microbial growth (Bashan, 1998).

Moreover, carriers need to be easy to manufacture and able to be mixed with other compounds like nutrients in order to provide a good environment for the live cells. In fact, they need to be easy to mix and easy to fabricate as they are intended to be used massively. Furthermore, they need to be easy to handle and store because they will be used by farmers who will use them periodically and will need to have reservoirs of the products for rapid use (Bashan, 1998). Nowadays, research has been focused on the creation of new carriers that can have all of these characteristics, and can provide positive results for the application of inoculants. Thus, a good carrier may not have all the characteristics cited, but it is recommended that it has as many as possible (Bashan, 1998).

Inoculants, which are formulated (carrier and cells) or unformulated (only cells) biological products can form aggregates that make them more resistant to environmental changes and can represent another type of inoculants (Burdman et al., 2000). In biocontrol, formulations can affect the performance of the biocontrol agent, so it is really
necessary to take into account these parameters when developing new formulations (Fravel, 2005).

*Opportunities for new microbial inoculants*

Despite the wide variety of PGPR, it has been found that the dominant bacteria belong to the genus *Pseudomonas* and *Bacillus*. Research related to these bacteria has lead to a better understanding of the mechanisms involved in plant growth promotion and biocontrol. However, there are other genera that have not been deeply studied including *Mitsuaria* and *Burkholderia* for example, which have shown to be active against soilborne pathogens in several crops (Benítez and McSpadden Gardener, 2009). In the case of *Burkholderia*, a few studies have shown its effectiveness as a biocontrol agent in tomato through the production of siderophores, in addition, some have found evidence of nitrogen fixation. It is very important to know which other mechanisms are involved in their activity, as well as their behavior around other biocontrol agents (Caballero-Mellado et al., 2007). Other examples are species from the *Burkholderia cepacia* complex, which have been used in biological control and bioremediation due to their capacity of degrading xenobiotic pollutants (O’Sullivan and Mahenthiralingam, 2005; Parke and Gurian-Sherman, 2001). However, some *Burkholderia* species, specifically *Burkholderia cepacia* have reported to be associated with cystic fibrosis in humans (Harman et al., 2010). Due to this fact, it is important to study the versatility of bacteria, specifically of *Burkholderia* species to avoid using potential human pathogens (Parke and Gurian-Sherman, 2001).
Other microorganisms have also proven their potential as biofertilizers and biocontrol agents; such is the case of algae based biofertilizers. The knowledge of this potential biofertilizer comes from the past decades, when producers used green manures to stimulate plant growth. In fact, dry green algae contain high percentages of macro and micro nutrients as well as amino acids. A preliminary study reported that seedling growth of lettuce plants increases when they are inoculated with green microalgae; the investigation also found higher fresh weight of the inoculated plants compared to a control (Faheed and Abd-el Fattah, 2008). Moreover, a kind of seaweed, known as kelp has been also used as biofertilizer and for biocontrol. For example, *Ecklonia maxima*, applied as soil drench in tomato, stimulated plant growth and reduced nematode infestation. This could be seen when plant fresh weight was measured and when nematode infestation was evaluated. When compared to an un-inoculated control, in both cases, inoculation of *Ecklonia maxima* stimulated greater fresh weight (Crouch and Staden, 1993). Thus, it is important to know that microbial variety can offer different types of inoculants depending on their activity. However, research should be focused on knowing their efficacy as potential biofertilizers and biocontrol agents.

Therefore, the hypothesis, objectives and approaches of this study were as follows:

Hypothesis to be tested for objective 1: Biocontrol and biofertilizer effects of experimental, commercial and pre-commercial microbial inoculants can be detected by developing an appropriate bioassay method.

Objective 1: Develop and test bioassay methods to assess microbial inoculants for plant growth promotion and disease suppression.
Approach: To develop an effective method that can be used for researchers and industry to test microbial inoculants effect in biocontrol and plant growth promotion.

Hypothesis to be tested for objective 2: *Mitsuaria* and *Burkholderia* experimental can effectively control disease in tomato and wheat.

Objective 2: Develop a series of bioassays with the aim of testing experimental strains *Mitsuaria* and *Burkholderia* for biocontrol and plant growth promotion

Approach: To apply a previously tested bioassay method to assess disease severity reduction and plant growth promotion of treatments prepared with the experimental bacterial strains and compare their effects with other commercial and pre-commercial microbial inoculants.
CHAPTER 2

Development and testing of bioassay methods to assess microbial inoculants for plant growth promotion and disease suppression

ABSTRACT

A series of experiments were conducted with the aim of testing bioassay techniques for detecting biofertilizer and biocontrol effects. Different microbial inoculants were used as treatments, including Mitsuaria sp. strain H24L5A, Burkholderia sp. strain R4F2, Algae-Based Biofertilizer (ABB), Abundance™ and commercial products Re-New™ and Re-Start™. Biofertilizer activity was successfully detected for Re-New™ and Abundance™ in greenhouse and field bioassays. Soil drenching was found to be the most effective technique for treatments inoculation in greenhouse bioassays leading to successful detection of biofertilizer capacity of H24L5A, R4F2 and Re-Start™. ABB showed a slight biofertilizer capacity in a wheat field trial. Both crops grew under greenhouse conditions, but variation in temperature ranges was detected, suggesting that heat stress may have been a problem. Unsuccessful detection of treatment effects in some experimental treatments may be attributed to the lack of replication of the bioassays as well as variation in the environmental conditions. The results of these sets of bioassays provided useful information for treatment application methods as well as bioassay techniques, which were applied in the next set of experiments.
INTRODUCTION

Efficiency in plant growth stimulation as well as in disease suppression is one of the most important factors that confer liability to products used in agriculture. In fact, farmers and growers base their selection of fertilizers and pesticides in terms of yield improvement and pest control effectiveness respectively. In the selection of products, farmers can find that their nature may vary; typically they can be derived from natural materials (animal, plants, bacteria and certain minerals) or have a chemical basis. In fact, the promising evidence of natural-based products such as plant growth promoting bacteria as biofertilizers and biopesticides has open the possibility of the implementation of them as part of some agricultural practices.

Accordingly, “plant strengthening agents”, “inoculants”, and “biofertilizers” are terms that apply to products and microorganisms sold to improve plant performance (Harman, 2010). Biopesticides, include naturally occurring substances, microorganisms and pesticidal substances produced by plants containing added genetic material that control pests. “Biofertilizer” is a recently coined term that commonly refers to the use of soil microorganisms to increase the availability and uptake of mineral nutrients for plants. Among these microorganisms, the most commonly used and available in the market include fungi, alga and bacteria. Fungal biological control agents provide considerable potential that is not often exploited for insect, disease and weed control (Butt, 2000). In addition, marine algae as a rich source of bioactive compounds have been used as biofertilizers leading to improve seed germination, higher yields and increased resistance to diseases (Mercier, 2001). Plant growth promoting rhizobacteria (PGPR) that grow in association with a plant host can stimulate its growth through direct and indirect
mechanisms. Direct mechanisms include 1) production of phytohormones, 2) solubilization of phosphates and 3) increase uptake of iron. Moreover, indirect effects can include antibiotic production, nutrient competition, parasitism, and inhibition of pathogen toxins or induced resistance (Vessey, 2003).

Biological control is the result of indirect plant growth promotion mechanisms. Bacterial biocontrol agents can induce resistance in plants by activating host defense mechanisms. This phenomenon can also happen without direct contact between the pathogen and the biocontrol agent, for example when it is applied to the roots, disease suppression is attributed to induced systemic resistance (Gnanamanickam, 2002). Indeed, biological control of plant pathogens by microbes in general is the action of suppressing pathogens to favor plants, a fact that has increased the interest especially of organic growers as an alternative of fungicides (Fravel, 2005).

Factors like the increase in cost of soil fumigation, concerns about fungicide exposure and development of insensitivity by pathogens to fungicides have contributed to the strong need to reduce dependence on chemicals to protect crops from diseases. As a result, ecologically-based pest management practices such as biological control are now being adopted. In addition, the environmentally and sustainable shift that agriculture and agri-food sectors are experiencing is leading the development of organic farming (Rigby and Cáceres, 2001; Fravel, 2005). In fact, organic farming systems rely on biological control as part of the practices for pest management and prohibit the use of synthetic chemicals in crop production. USDA estimates that in the US organic farming has been one of the fastest growing segments in the last decade, leading to a 15% increase in the number of organic farmers. In Ohio, according to the latest data between 2006 and 2008,
the total acres used for organic agriculture increased from 37,710 to 52,949 (USDA, 2010). Based on these facts and the need for environmentally friendly microbial technologies for sustainable agriculture, the use of microbial biocontrol agents has increased over the last 40 years (Fravel, 2005).

The success of biocontrol depends on the screening process for the biocontrol agent, as well as its ability to survive in different environments, correct formulation and type of application to the plant or soil and more importantly consistency on field trials, which means that the biocontrol agent is able to perform in the field as well as it did in the greenhouse (Fravel, 2005). First, as described by Benitez and McSpadden Gardener (2009), there are multiple approaches to screen biocontrol agents, which range from genomic comparative studies to culture-based screenings. Early methods focused on recovering the cells and then screen them for activity. Benitez and McSpadden Gardener (2009) developed an applied culture-independent molecular tool to first identify and then recover biocontrol bacteria, leading to the isolation *Burkholderia* sp. and *Mitsuaria* sp. from disease suppressive soils. Moreover, other approaches for isolation of biocontrol agents include basic principles of phenomenon visualization. The Algae-Based Biofertilizer, a unique mixed culture of green algae and bacteria was isolated from the surface of a low carbon, sand based growth medium (*unpublished*, S. Park and B. McSpadden Gardener). Second, survival in different environments is related to activity in-vitro and in-vivo tests under controlled conditions and also under field conditions. To understand biocontrol agents’ mechanism or other desirable traits, they can be grown on different substrates under different environments and edaphic conditions (Gnanamanickam, 2002). Correct formulation and delivery play an important role at the
time of applying the biological control agents. Delivery systems that take into account
time and place should be well developed as well as application methods, which are very
important to guarantee biocontrol agent establishment, proliferation and activity. For
instance, researchers nowadays are paying attention to basic but important facts on
application such as applying the agents as seed coat, sprays or as soil-drench or by root-
dipping (Fravel, 2005; Xue et al., 2009). Finally, to test the specificity of the biocontrol
agents to a location or a pathogen, it is necessary to conduct tests to assess their
potentiality in order to exploit them. Once identified the biocontrol agent and its
biocontrol potential, it needs to go through greenhouse and field testing.

Greenhouse and field testing has three principal objectives: 1) select active
biocontrol agents that control a spectrum of plant pathogens, 2) evaluate the biocontrol
agents under controlled environmental conditions and 3) evaluate different formulations
and application methods. All scenarios are influenced by the type of biocontrol agent,
environmental conditions, kind of cultivars, type of formulation, application method and
method and time of disease assessment. Biocontrol agents can be applied as seed
treatments, as root-dipping solutions or directly to the soil (Gnanamanickam, 2002; Xue
et al., 2009). Application method will depend on the type of target pathogen, stage of the
crop, nature and spread of disease and climatic conditions. Soil application could provide
protection from the nurseries until after transplanting (Gnanamanickam, 2002).
Nowadays biocontrol agents are being tested directly in the system they are intended to
be used in and not only on experiments done in vitro or in greenhouses (Fravel, 2005).

The main goal of this study was to develop bioassay methodologies to assess
biofertilizer and biocontrol effects under greenhouse and field conditions. Biofertilizer
effect was determined by assessing shoot height, shoot weight, root height and shoot weight and biocontrol capacity was determined by scoring disease severity. It was hypothesized that the inoculants tested could enhance plant growth and could provide disease control in a detectable scale.

MATERIALS AND METHODS

Plant material, pathogens and inoculants

Seeds of hybrid tomato (*Solanum lycopersicum* ‘Celebrity F1’) (Johnny’s Selected Seed, Winslow, ME) were hot water-treated (Miller and Lewis, 2005), air dried, packaged in clean plastic Petri dishes. Seeds of organic ‘Hard Red RB07’ spring wheat (*Triticum aestivum*) (Albert Lea Seed House, Albert Lea, MN) were used in this study. *Xanthomonas gardneri* strain SM230-10, causal agent of bacterial leaf spot was provided by Dr. Sally Miller laboratory (Wooster, OH) (Ma et al, 2011).

The inoculants tested in the sets of bioassays in this study, are described in Table 2.1; in addition, tap water was applied as a negative control (NC). Re-Start™ (Regen Earth, Cincinnati, OH) *Ecklonia maxima* kelp concentrate biostimulant was prepared at a ratio of 1:200 in autoclaved distilled water. Re-New™ (Regen Earth, Cincinnati, OH) a liquid of marine fish and fresh kelp concentrate biostimulant was prepared at a ratio of 1:500 in autoclaved distilled water. Abundance™, an experimental and pre-commercial product composed of organic materials (1.18 g kaolin clay, 0.39 g dried yucca extract, 0.39 g lime, 0.39 g of yeast extract and 0.2 g of freeze-dried bacteria (from the genus *Pseudomonas* sp. and *Bacillus* sp.) per 500 ml of water, was dissolved in autoclaved distilled water according to manufacturer’s instructions. Algae-based
biofertilizer (ABB), a mixed culture of green algae and bacteria belonging to the order *Chlamydomonadales* and *Microbacterium* spp. was prepared at a concentration of $10^6$ cells/mL ($\text{OD}_{595} = 0.05$) was streaked from a glycerol stock into BG-11 medium (Atlas, 1995) plates and allowed to grow with in an incubator at 25°C lighted with 450 lumens for 8 days. Afterwards, a loopfull of the culture was added to a 1X BG-11 liquid medium flask with airline tubing attached to airstones and connected to an air pump and placed in a room with light at 25°C for 8 days. Before inoculation, ABB culture was centrifuged at 8000 RPM for 10 minutes, supernatant was discarded and precipitated cells were re-suspended in water to obtain a final $\text{OD}_{595}$ of 0.05 ($10^6$ cells/ml) using an Allegra 21R refrigerated centrifuge (Beckman Coulter, Brea CA). As negative control, sterilized distilled water was used. Bacterial cultures of *Mitsuaria* sp. strain H24L5A and *Burkholderia* sp. strain R4F2 were maintained in King’s broth B (DIFCO Laboratories, Detroit, MI) in 30% glycerol at -80°C, fresh cultures were prepared from the frozen stock on 1/10 Tryptic Soy Agar plates (DIFCO Laboratories, Detroit, MI) and allowed to grow for 48 hours at room temperature. A full loop from each culture plate was then added to 500 ml flasks containing 350 ml 1/5 X Tryptic Soy Broth (DIFCO Laboratories, Detroit, MI), incubated at 29°C and shaken at 75 RPM for 24 hours. Cell density was determined using a haemocytometer and concentrations of $10^4$, $10^6$, $10^7$ and $10^8$ cells/ml were determined. Final dilutions were made using a solution of 1/5 Tryptic Soy Broth prepared in distilled autoclaved water. Water was applied as a negative control (NC).

*Development of biofertilizer and biocontrol bioassays under greenhouse conditions*
For the first set of bioassays, tomato and wheat seeds were sown in Whitney Farms Organic Potting Soil (Scotts Company, Marysville, OH) contained in plastic cone-tainers of 164 ml of volume, 3.8 cm diameter and 21 cm depth (Stuewe & Sons, Inc., Tangent, OR). A randomized complete block design, consisting of five treatments inoculated with five replicates each was used. One tomato seed and two wheat seeds were sown in each cone-tainer; the second wheat seedling was removed after emergence. Plant growth promotion was evaluated in two experiments of tomato and in four experiments of wheat; biocontrol capacity was evaluated in two other experiments of tomato.

Experimental, commercial and pre-commercial inoculants used in these set of bioassays were: Re-Start™, Re-New™, Abundance™ and Algae-Based Biofertilizer, according to Table 2.1 they corresponded to: MIC2 1X, MIC1 1X, MIF1 1X, MIE3 1X, in addition, water was applied as negative control (NC). All these treatments were prepared according to Table 2.1. To determine the total volume of treatments needed, three cones were filled with starting mix and drenched with a volume of 20, 40 and 50 ml of water. The experiment was repeated until water flowed through the bottom for more than 10 minutes. The final volume to be used was 50ml per cone for the drench treatments and for daily watering. After filling the cone-tainers and sowing the seeds, treatments were immediately applied. Cones were covered with plastic immediately after being treated and removed after seedling germination.

Treatments were applied twice during the entire duration of the bioassay; the second application was four weeks after the first one. In the tomato biocontrol bioassays, _X. gardneri_ strain SM230 was sprayed at a final concentration of $10^8$ cells/ml to one single leaf per seedling as a cell suspension in 1/3 King’s broth B (DIFCO Laboratories,
Detroit, MI). Challenged seedlings were about 3-5 inches tall and had 2-6 true leaves developed. All inoculated leaves were covered with plastic bags for 36 hours. After incubation time, bags were removed and challenged leaves were tagged with a wire.

Symptom development of bacterial spot (i.e. chlorotic haloes, yellowing as well as curly leaves and presence of small brown lesions) was monitored daily and disease severity was recorded 7 days after pathogen inoculation using a 0 to 5 rating scale as follows: 0 = healthy leaf, 1 = up to 20% of the leaf showing symptoms, 2 = 40% of the leaf showing symptoms, 3 = 60% of the leaf showing symptoms, 4 = 80% of the leaf showing symptoms and 5 = all the leaf showing symptoms. Six weeks after the first application of treatments, shoot height, shoot weight, root length and root weight of tomato and wheat plants were assessed to determine the effects on plant growth promotion of the different treatments. To recover the entire plants, cone-tainers were cut by using a scalpel. Plants were then shaken to remove organic potting mix from the roots; roots were cut from the stem basis and shoot and rood fresh weight were recorded.

For the second set of bioassays, tomato seeds were sown in an inorganic, chemically, stable growing medium composed by weight of: 60% industrial grade 20:40 silica sand (Best Sand, Chardon, OH) and 40% Primera One calcinated clay field conditioner (Profile Products, LLC, Buffalo Grove, IL). The two dry components of the medium were mixed prior to fill the cone-tainers. In addition, 50 ml of a modified Hoagland’s solution consisting of reagent grade 0.5 mol/l KCl, 0.5 mol/ L K₂SO₄, 0.5mol/l MgSO₄·7H₂O, 0.05 mol/l Ca(H₂PO₄)₂·2H₂O, 0.01 mol/l CaSO₄·2H₂O and 0.5 mol/l KH₂PO₄ along with a solution of 10 ppm of NH₄NO₃ diluted in distilled water were added weekly to each plant (Bumgarner et al., 2012). Tomato seedlings were first sown
on seedling trays, then each seedling was transplanted into a single cone-tainer (Stuewe & Sons, Inc., Tangent, OR). Two treatment application methods were assessed: soil-drenching and root-dipping. A randomized complete block design per application method was carried out. The treatments applied in this second set of bioassays were: *Mitsuaria* sp. strain H24L5A, *Burkholderia* sp. strain R4F2, Re-Start™, Algae-Based Biofertilizer (ABB). According to Table 2.1 they corresponded to: MIE1 104, MIE1 106, MIE1 107, MIE2 104, MIE2 106, MIE2 108, MIC2 ½X, MIC2 1X, MIC2 2X, MIE3 ½X, MIE3 1X and MIE3 2X, in addition, water was applied as negative control (NC). For soil drenching, 50 ml of each treatment were applied per cone, volume defined previously in the first set of bioassays. Treatments were drenched into the growing medium containing the transplanted seedling. For root-dipping, seedlings were placed in 10mL of a different treatment for 3 minutes and then transplanted. Plant growth was evaluated 6 weeks after transplanting by recording shoot height and shoot weight.

*Development of biofertilizer and biocontrol bioassays under field conditions*

A tomato field bioassay testing the following treatments: Algae-Based Biofertilizer (ABB) and Re-New™ was carried out. According to Table 2.1, treatments corresponded to: MIE3 1X and MIC1 1X, in addition, water was applied as negative control (NC). Tomato seedlings were sown in organic potting soil (Better-Gro Pot-n-gro, Better-Gro LTD, Millersburg, OH), placed in an organic greenhouse and watered daily until they reached V2 growth stage; seedlings then were transplanted into the field. Treatments were applied by soil-drenching at a volume of 100 ml per plant at transplanting and a second application took place two weeks after transplanting. The field
bioassay consisted of a randomized complete block design with three treatments and eight replicates; the experimental unit was a single tomato plant. Each block consisted of two beds with three treatments per bed randomized using a random digits table (Forthofer et al, 2007). Plants were separated by 2 feet; distance between the beds was of 10 feet. Irrigation tape was laid in the center of each bed, which was covered with black plastic mulch. After 48 hours of the second application of treatments, *X. gardneri* strain SM230 was prepared at two different concentrations: a high concentration (HC) of $2.88 \times 10^7$ cells/ml and a low concentration (LC) of $2.88 \times 10^4$ cells/ml. Two different fully extended leaves were sprayed with *X. gardneri* suspensions; one leaf was inoculated with LC and the other one with HC. Leaves were covered with plastic bags for 18 hours, and then challenged leaves were tagged with a wire. Symptom development of bacterial spot (chlorotic haloes, yellowing as well as curly leaves and presence of small brown lesions) was monitored daily and disease severity was recorded 7 days after pathogen inoculation as percentage of diseased leaf that ranged from 0 to 100%. Plant growth evaluation was carried out by recording plants’ height and shoot biomass of one plant per treatment corresponding to each block six weeks after transplanting.

In addition, a winter wheat field bioassay testing the following treatments: Algae-Based Biofertilizer (ABB), Abundance™ and Re-Start™ was carried out as well. According to Table 2.1 treatments corresponded to: MIE3 1X, MIF1 1X and MIC2 1X, in addition, water was applied as negative control (NC). A randomized complete block design consisting of a 100 ft long plot with 10 feet of width, with two sides of five rows 100 ft long of winter wheat, was divided into five blocks of 100 sq ft alternating between the two sides. Each treatment was applied into plots of 20 sq ft with 5 rows. MIE3 1X
and NC were applied by soil drenching and MIF1 1X and MIC2 1X were sprayed directly to base of shoots at a volume of 130 ml per row. Winter wheat plants were inoculated at the stage of jointing, when they were approximately 30 cm tall. All the treatments were applied twice; the second application took place four weeks after first one. Height was evaluated four weeks after first application and the number of heads per meter was counted after six weeks. To count heads, a 1m-stick ruler was lied out on the ground in the middle of each plot then the wheat heads on the plants along the ruler were counted and reported as heads per meter.

Statistical analysis

Analyses of variance were run on MINITAB® (v16.1.0 Minitab Inc., State College, PA) using a General Linear Model and pairwise comparisons of means were performed based on Tukey’s Honestly Significant Difference (HSD) test.

RESULTS

Biocontrol and plant growth promotion greenhouse bioassays

In the experiments conducted in tomato and wheat using an organic potting soil, a fertilizer effect of the microbial inoculants was detected when shoot height, shoot weight and root weight were assessed ($P<0.05$). The application of MIC1 1X and MIF1 1X increased shoot height by 20 to 48% in tomato and by 23% in wheat. Both treatments increased shoot weight by 150 to 200% in tomato and by 80 to 140% in wheat. Both treatments also increased root weight by 33 to 100% in tomato and 44 to 60% in wheat. The rest of the treatments provided less than 20% of plant growth parameters increase,
showing no fertilizer effect to the tomato and wheat plants. No biocontrol effect was detected for the rest of the treatments ($P>0.05$). In addition, no significant evidence of biocontrol in tomato and wheat was found for any of the treatments ($P>0.05$) (Table 2.2 and Table 2.3).

In another set of the experiments conducted in tomato in an inorganic matrix, there was significant evidence of effects of the methods of application on plant growth promotion ($P<0.05$). Soil-drenching application of bacterial cells as treatments MIE1 107 and MIE2 108 led to greater values of shoot height and root length when compared to root-dipping application of the same treatments ($P<0.05$) (Table 2.4). The application of MIE1 107 stimulated greater tomato growth compared to the NC since shoot height and shoot weight were increased significantly by up to 100% in plants corresponding to soil-drenching and by up to 37% in plants corresponding to root-dipping. MIE2 108 stimulated tomato growth as well, compared to the NC since it significantly increased shoot height by 94% and shoot weight by 300% in plants corresponding to soil-drenching method ($P <0.05$). The application of MIC2 in all its concentrations also stimulated tomato growth compared to the NC, since shoot height was increased significantly by 22 to 37% in soil drenching, and by 20% in root dipping. Shoot weight was also increased 100% in soil drenching, and by 50% in root dipping. Biofertilizer effect detected for MIE3 did not reach 10% in any of its concentrations (Table 2.4).

**Biocontrol and plant growth promotion field bioassays**

In the tomato field bioassay, no biocontrol effect was detected due to the very high disease pressure. However, biofertilizer effect was seen even though there was no
significant evidence of the effects of microbial inoculants on biocontrol and biofertilizer
effects ($P>0.05$). A trend followed by the application of MIC1 1X stimulating tomato
growth with the highest values of shoot height, shoot weight and root weigh was seen
(Table 2.5).

In the wheat field bioassay, there was no significant evidence of the effects of
microbial inoculants on plant growth ($P>0.05$). However, there was a trend followed by
the application of MIF1 1X increasing wheat growth by 2% and MIE3 1X increasing the
number of heads/m by 3% (Table 2.6).

Temperature Measurements

Temperature patterns inside the greenhouse where the second set of bioassays
with an inorganic growing medium was used fluctuated between a minimum 17°C and a
maximum of 52°C. During the first two weeks, temperatures reached up to 52°C, in the
next following weeks, maximum temperatures reached 40°C (Figure 2.1).

DISCUSSION

The bioassay methodologies used effectively detected differences among the
treatments applied in the bioassays (Table 2.2, Table 2.3, Table 2.5). Biofertilizer effect
of Re-New™ was detected as expected in greenhouse and field bioassays. The favorable
response of the tomato and wheat plants to this treatment may be due to its high content
of potassium, sodium, calcium, phosphorous and magnesium. These components
provided plants with available nutrients to enhance their growth. In addition the
concentrated blend of kelp and marine fish emulsion could have stimulated root growth
as kelp synthesizes phytohormones and fish emulsions could have had fertilizer
assessed the capacity of fish emulsion as a source of inorganic nutrients on radish and
found that fish emulsion can also be a substrate for microbes that produce plant growth
regulators. Our data suggests that fish emulsion mixed with kelp could have enhanced
wheat and tomato growth. In addition, the components of fish emulsion (inorganic
elements, mixtures of essential amino acids, lipids, significant amounts of riboflavin,
pantothenic acid, niacin, biotin, folacin and vitamin B-12) could have been absorbed by
the roots providing growth enhancement (reviewed by El-Tarabily et al., 2003).

Biofertilizer effect was also detected for H24L5A and R4F2 strains belonging in
the soil-drenching and root-dipping application techniques (Table 2.4). Significant results
were seen in both application methods but clear differentiation was shown in the soil-
drenching applications, which suggest that soil-drenching is a more effective method than
root dipping for the application of bacterial strains. This is an agreement with the results
showed by Xue et al (2009), who after assessing root-dipping and soil-drenching of
Acinetobacter and Enterobacter treatments on tomato found that soil-drenching led to
higher levels of colonization than root-dipping. Even though colonization was not
assessed in these experiments, plant growth is attributed to greater concentration of cells
applied when drenching the treatments compared to the root-dipping, where less cells
attach to the roots (Table 2.4). Biofertilizer capacity of H24L5A and R4F2 treatments
may also be attributed to the nitrogen provided by the culture medium (1/5 Tryptic Soy
broth). In addition, soil drenching has proved to be an effective method for Re-Start™
application (Table 2.4). As described by an experiment performed Crouch and van
Staden, (1992), where *Ecklonia maxima* showed significant effect on plant growth by enhancing plants fresh weight when applying by soil-drenching due to its capacity of synthesizing cytokinins As values led by Re-Start™ were greater than those for ABB, we can speculate that the concentration of *Ecklonia maxima* cytoplasm and its content of phytohormones may have stimulated plant growth more effectively than ABB under the conditions of these experiments.

Biofertilizer effect was also detected for Abundance™ (MIF1 1X) in the tomato greenhouse bioassay as well as in the wheat field bioassay (Table 2.2, Table 2.3 and Table 2.6). This treatment is composed by: kaolin clay, dried yucca extract, lime, yeast extract and freeze-dried bacteria (*Pseudomonas* sp. and *Bacillus* sp.) has proved to be a stable formulation that significantly enhanced tomato growth. This effect could have been attributed to the biological and chemical components of the powdered formulation. The first component, kaolin clay, has been proved to be used as enhancer on the performance of some microbial products. Previous studies have shown that kaolin clay led to a better shelf life of *Trichoderma harzianum* formulations, as it retained viable propagules up to 90 days (Prasad and Rangeshwaran, 2000). Results in the bioassays suggest that kaolin could have contributed to the protection of the dry *Pseudomonas* sp. and *Bacillus* sp. cells contained in the formulation, which could have helped in the induction of tomato and wheat growth. As kaolin is insoluble in water, it could form a protection barrier when applied in plants, which is why it is used as pesticide by growers (EPA, 2012). For reference, kaolin clay is composed by total kaolinite 97%: silicon oxide 46%, aluminum oxide 37%, iron oxide 0.79% and titanium oxide 0.37%, and has other elements such as phosphorus, potassium, calcium, sodium, magnesium, etc. in
lower quantities (Badmus and Olatinsu, 2009). In addition, dried yucca extract, derived from the plant *Yucca schidigera*, used as a sticking agent could have helped in the adherence of the formulation to the roots and consequently in the better performance of the treatment when promoting plants growth. Lime, as a calcium-source material, defined as calcium carbonate, was a source of calcium for plants and could have improved the uptake of essential nutrients. Yeast extract, as a source of nutrients like magnesium, zinc, iron, copper among others, amino acids and glucans, represents a nutrition source for the microorganisms and the plants (Grant and Pramer, 1962; Brower, 2011). In this case, unspecified freeze-dried bacteria from the genus *Pseudomonas* sp. and *Bacillus* sp., were added, they are known to be used as a wastewater treatment, and proved to be non-pathogenic (Osprey Biotechniques, 2012). Furthermore, in the field bioassay on wheat, we can only speculate that the application of ABB could slightly increase wheat head number. This may represent the first attempt to use ABB in a field experiment as a potential biofertilizer of wheat.

No significant results found in some of the bioassays could be due to the lack of replication, lack of effectiveness of the treatment per se or due to variation on environmental conditions as temperature (Figure 2.1). In this set of bioassays, temperature reached high peaks, exceeding the maximum temperature tolerated by wheat (30°C-32°C) and tomato (32°C), which could have influenced the development of the different treatments (Gould, 1983; Khan and Shewry, 2009). In conclusion, the results of this set of bioassays provided valuable information for the development of the next set of experiments described in the next chapter.
<table>
<thead>
<tr>
<th>Microbial inoculant</th>
<th>Type</th>
<th>Description</th>
<th>Rate</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mitsuaria</em> sp. strain H24L5A</td>
<td>Experimental 1</td>
<td>Betaproteobacteria isolated from disease suppressive soils, applied as cell suspension on 1/5 Tryptic Soy Broth</td>
<td>$10^4$ cells/ml</td>
<td>MIE1 104</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10^6$ cells/ml</td>
<td>MIE1 106</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10^7$ cells/ml</td>
<td>MIE1 107</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp. strain R4F2</td>
<td>Experimental 2</td>
<td>Betaproteobacteria isolated from disease suppressive soil applied as cell suspension on 1/5 Tryptic Soy Broth</td>
<td>$10^4$ cells/ml</td>
<td>MIE2 104</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10^6$ cells/ml</td>
<td>MIE2 106</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10^8$ cells/ml</td>
<td>MIE2 108</td>
</tr>
<tr>
<td>Algae-Based Biofertilizer (ABB)</td>
<td>Experimental 3</td>
<td>Mixed culture composed of green algae and bacteria, isolated from the surface of a low carbon, sand-based growth medium, applied as liquid suspension</td>
<td>$10^5$ CFU/ml</td>
<td>MIE3 ½X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10^6$ CFU/ml</td>
<td>MIE3 1X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10^8$ CFU/ml</td>
<td>MIE3 2X</td>
</tr>
<tr>
<td>Re-New™</td>
<td>Commercial 1</td>
<td>Concentrated blend of <em>Ecklonia maxima</em> cytoplasm and marine fish emulsion diluted with water</td>
<td>2 ml/l</td>
<td>MIC1 1X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.5 ml/l</td>
<td>MIC2 ½X</td>
</tr>
<tr>
<td>Re-Start™</td>
<td>Commercial 2</td>
<td><em>Ecklonia maxima</em> cytoplasm concentrate diluted with water</td>
<td>5 ml/l</td>
<td>MIC2 1X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 ml/l</td>
<td>MIC2 2X</td>
</tr>
<tr>
<td>Abundance™</td>
<td>Pre-commercial 1</td>
<td>Powder formulation composed of <em>Pseudomonas sp.</em> and <em>Bacillus sp.</em> freeze-dried cells, kaolin clay, dried plant extract and lime, diluted with water</td>
<td>5.1 g/L</td>
<td>MIF1 1X</td>
</tr>
</tbody>
</table>

Table 2.1. Experimental microbial inoculants with a description of the rate and codes of the different treatments used in the tomato and wheat greenhouse bioassays with the objective to assess their biocontrol and plant growth promotion capacity.
### Treatment Response variables

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease Severity&lt;sup&gt;y&lt;/sup&gt;</th>
<th>Height&lt;sup&gt;y&lt;/sup&gt; (cm)</th>
<th>Weight&lt;sup&gt;y&lt;/sup&gt; (g)</th>
<th>Length&lt;sup&gt;y&lt;/sup&gt; (cm)</th>
<th>Weight&lt;sup&gt;y&lt;/sup&gt; (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIE3 1X</td>
<td>3.8 a</td>
<td>6.9 c</td>
<td>0.4 c</td>
<td>19.1 ab</td>
<td>0.4 c</td>
</tr>
<tr>
<td>MIC1 1X</td>
<td>3.5 a</td>
<td>12.1 a</td>
<td>2.0 a</td>
<td>21.5 a</td>
<td>1.2 a</td>
</tr>
<tr>
<td>MIC2 1X</td>
<td>3.2 a</td>
<td>8.3 c</td>
<td>0.6 bc</td>
<td>18.2 b</td>
<td>0.5 bc</td>
</tr>
<tr>
<td>MIF1 1X</td>
<td>4.0 a</td>
<td>10.0 b</td>
<td>0.9 b</td>
<td>20.1 ab</td>
<td>0.8 b</td>
</tr>
<tr>
<td>NC</td>
<td>3.3 a</td>
<td>8.2 c</td>
<td>0.6 bc</td>
<td>20.1 ab</td>
<td>0.6 bc</td>
</tr>
</tbody>
</table>

| P-value<sup>z</sup> | 0.5 | <0.05 | <0.05 | <0.05 | <0.05 |

Table 2.2. Biocontrol and biofertilizer effects of experimental and commercial microbial inoculants in greenhouse evaluations in tomato.

<sup>y</sup> Values are the means of two experiments with five replicates each. Tukey’s multiple comparison results are presented as a grouping table, treatments that share a letter are not significantly different.

<sup>z</sup> P-values correspond to the ANOVA tables at an alpha value set at 0.05.

### Treatment Response variables

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot Height&lt;sup&gt;y&lt;/sup&gt; (cm)</th>
<th>Shoot Weight&lt;sup&gt;y&lt;/sup&gt; (g)</th>
<th>Root Length&lt;sup&gt;y&lt;/sup&gt; (cm)</th>
<th>Root Weight&lt;sup&gt;y&lt;/sup&gt; (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIE3 1X</td>
<td>27.9 bc</td>
<td>0.5 c</td>
<td>22.9 a</td>
<td>1.2 ab</td>
</tr>
<tr>
<td>MIC1 1X</td>
<td>34.7 a</td>
<td>1.2 a</td>
<td>24.7 a</td>
<td>1.5 a</td>
</tr>
<tr>
<td>MIC2 1X</td>
<td>25.9 c</td>
<td>0.4 c</td>
<td>22.8 a</td>
<td>0.9 b</td>
</tr>
<tr>
<td>MIF1 1X</td>
<td>32.0 ab</td>
<td>0.9 b</td>
<td>23.4 a</td>
<td>1.3 ab</td>
</tr>
<tr>
<td>NC</td>
<td>28.1 bc</td>
<td>0.5 c</td>
<td>23.5 a</td>
<td>0.9 b</td>
</tr>
</tbody>
</table>

| P-value<sup>z</sup> | <0.05 | <0.05 | 0.241 | <0.05 |

Table 2.3. Biofertilizer effect in shoot height, shoot weight, root length and root weight of wheat in greenhouse experiments using an organic potting soil.

<sup>y</sup> Values correspond to the means of four experiments with five replicates each. Tukey’s multiple comparison results are presented as a grouping table, treatments that share a letter are not significantly different.

<sup>z</sup> P-values correspond to the ANOVA tables at an alpha value set at 0.05.
Table 2.4. Effect of different concentration of inoculants and application techniques on tomato growth in greenhouse experiments.

x The symbol ‘-‘ corresponds to missing plants, lost after transplanting.

y Values correspond to the means of five replicates per treatment. Tukey’s multiple comparison results are presented as a grouping table, treatments that share a lower case letter in the same column and an upper case letter regardless the column and the row, are not significantly different.

z P-values correspond to the ANOVA tables at an alpha value set at 0.05.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Height&lt;sup&gt;y&lt;/sup&gt; (cm)</th>
<th>Weight&lt;sup&gt;y&lt;/sup&gt; (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drenching</td>
<td>Dipping</td>
<td>Drenching</td>
</tr>
<tr>
<td>MIE1 104</td>
<td>-</td>
<td>4.6 abc</td>
</tr>
<tr>
<td>MIE1 106</td>
<td>-</td>
<td>4.8 abc</td>
</tr>
<tr>
<td>MIE1 107</td>
<td>8.4 a A</td>
<td>5.9 a</td>
</tr>
<tr>
<td>MIE2 104</td>
<td>-</td>
<td>4.8 abc</td>
</tr>
<tr>
<td>MIE2 106</td>
<td>-</td>
<td>4.8 abc</td>
</tr>
<tr>
<td>MIE2 108</td>
<td>6.8 a B</td>
<td>4.9 abc</td>
</tr>
<tr>
<td>MIE3 ½X</td>
<td>3.5 bc</td>
<td>DEFGH</td>
</tr>
<tr>
<td>MIE3 1X</td>
<td>2.5 c H</td>
<td>3.0 d</td>
</tr>
<tr>
<td>MIE3 2X</td>
<td>3.2 bc</td>
<td>DEFGH</td>
</tr>
<tr>
<td>MIC2 ½X</td>
<td>4.5 b</td>
<td>CDEFG</td>
</tr>
<tr>
<td>MIC2 1X</td>
<td>4.3 b</td>
<td>CDEFG</td>
</tr>
<tr>
<td>MIC2 2X</td>
<td>4.8 b</td>
<td>CDEF</td>
</tr>
<tr>
<td>NC</td>
<td>3.5 bc</td>
<td>FGH</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P-value for treatment&lt;sup&gt;z&lt;/sup&gt;</th>
<th>&lt;0.05</th>
<th>&lt;0.05</th>
<th>&lt;0.05</th>
<th>&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-value for method&lt;sup&gt;z&lt;/sup&gt;</td>
<td>0.851</td>
<td></td>
<td>0.638</td>
<td></td>
</tr>
</tbody>
</table>
### Table 2.5. Biocontrol and biofertilizer effects of experimental and commercial microbial inoculants in a field evaluation in tomato.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Response variables</th>
<th>Disease Severity (%)</th>
<th>Shoot (cm)</th>
<th>Root (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low Concentration</td>
<td>High Concentration</td>
<td></td>
</tr>
<tr>
<td>MIE3 1X</td>
<td></td>
<td>93.8 a</td>
<td>98.8 a</td>
<td>57.6 a</td>
</tr>
<tr>
<td>MIC1 1X</td>
<td></td>
<td>100.0 a</td>
<td>98.8 a</td>
<td>57.9 a</td>
</tr>
<tr>
<td>NC</td>
<td></td>
<td>98.8 a</td>
<td>98.8 a</td>
<td>54.0 a</td>
</tr>
</tbody>
</table>

P-value\(^z\) 0.332 1.00 0.36 0.05 0.74

\(^y\) Values correspond the means of eight replicates per treatment. Tukey’s multiple comparison results are presented as a grouping table, treatments that share a letter are not significantly different.

\(^z\) P-values correspond to the ANOVA tables at an alpha value set at 0.05.

### Table 2.6. Effects of microbial inoculants on height and heads/m of wheat in a field bioassay.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Response variables</th>
<th>Height (cm)</th>
<th>Heads/m (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIE3 1X</td>
<td></td>
<td>74.3 a</td>
<td>117.0 a</td>
</tr>
<tr>
<td>MIC2 1X</td>
<td></td>
<td>74.1 a</td>
<td>114.0 a</td>
</tr>
<tr>
<td>MIF1 1X</td>
<td></td>
<td>75.0 a</td>
<td>115.0 a</td>
</tr>
<tr>
<td>NC</td>
<td></td>
<td>73.5 a</td>
<td>111.8 a</td>
</tr>
</tbody>
</table>

P-value\(^z\) 0.84 0.73

\(^y\) Values are the means of five replicates per treatment. Tukey’s multiple comparison results are presented as a grouping table, treatments that share a letter are not significantly different.

\(^z\) P-values correspond to the ANOVA tables at an alpha value set at 0.05.
Figure 2.1. Daily maximum and minimum temperatures (°C) recorded in the greenhouse during the development of bioassays with tomato and wheat with an inorganic growing medium using root-dipping and soil drenching.
CHAPTER 3

Inoculants containing *Mitsuaria* sp. strain H24L5A or *Burkholderia* sp. strain R4F2 show evidence of plant growth promotion and induced disease suppression

ABSTRACT

Experiments were conducted to determine the effects of inoculant treatments prepared with *Mitsuaria* sp. strain H24L5A and *Burkholderia* sp. strain R4F2 on plants. In the greenhouse bioassays, the application of H24L5A and R4F2 as re-suspended cells reduced the severity of bacterial leaf spot on tomato ($P<0.05$) by an average of 39% and 23% respectively. In contrast, the application of Actinovate®, a commercial microbial inoculant, showed a trend towards reduction of bacterial leaf spot of up to 25% on average in greenhouse. This observed trend suggests that H24L5A may provide greater disease reduction than a commercial inoculant, a trend also seen in a field trial. In addition, the application of H24L5A and R4F2 as spent medium and cell suspensions increased tomato and wheat growth by 20% to 64% in average, increase was statistically significant in shoot height and shoot weight of tomato experimental trials. Other experimental, commercial and pre-commercial microbial inoculants tested did not provide significant disease reduction or plant growth promotion, most of them showing less than 20% of disease reduction and plant growth increase on average. This is the first report of *Mitsuaria* sp. strain H24L5A and *Burkholderia* sp. strain R4F2 as biocontrol agents of bacterial leaf spot and plant growth promotion agents of tomato and wheat.
INTRODUCTION

In agriculture, the use of microbial inoculants with beneficial properties is likely to increase as an alternative and/or complement of chemicals. Microbial inoculants can be considered as safe, low cost and comfortable additives that represent a nature-based option for plant growth promotion and disease control (van Elsas et al., 1998; Al-Taweil et al., 2010). Plant growth promotion mediated by beneficial microbes, specifically bacteria can be traced back for centuries. The first patented product was registered about 100 years ago, using *Rhizobium* sp. as active ingredient. In addition, back in the 1930s and 1940s inoculation with nonsymbiotic rhizosphere bacteria like *Azotobacter* started and revived in the late 1970s (Bashan, 1998). On the other hand disease control mediated by microbes is relatively new as the first bacterial product used as a biocontrol agent was registered by the U.S Environmental Protection Agency (EPA) in 1979 (Fravel, 2005). Nowadays, according to the most updated list of EPA, a total of 35 bacterial strains are registered as biopesticides with the majority of them belonging to the genus *Bacillus*. Other genres like *Pseudomonas, Agrobacterium, Streptomyces, Burkholderia* and *Paecilomyces* are also included in the list and are sold commercially (EPA, 2012). The fact of having fewer different species of bacteria currently marketed as microbial pesticides, despite the improvement on the screening tools for microorganisms associated with plant pathogen suppression, is mainly due to lack of logical comprehensive testing and lack of information about registration. Two recently discovered strains, *Burkholderia* sp. R4F2, and *Mitusaria* sp. H24L5A identified from disease suppressive soils by the technique of terminal restriction fragment (TRF) length polymorphism (T-
RFLP) of bacterial 16S rRNA genes associated with damping-off disease suppression, have been used in this research (Benitez and McSpadden Gardener, 2008). These two strains that belong to the β-subclass of Proteobacteria are going through the early process of testing and formulation as promising biocontrol agents.

The genus *Burkholderia* belongs to the family *Burkholderiaceae* in the order *Burkholderiales*, it was proposed as a new genus that separated it from the *Pseudomonas* by Yabuuchi et al (1992). It is known for its ability to adapt to different habitats ranging from freshwater sediments to plant tissues. Sequencing of more than 35 genomes of *Burkholderia* over the past few years has provided very useful information about the pathogenicity and the biotechnological potential of this genus (Paganin et al., 2011). In general, *Burkholderia* can be divided in two major groups, the first one includes pathogens like the *Burkholderia cepacia* complex and the second one includes environmental non-pathogenic species mostly associated with plants. Indeed, among the second group there are interesting properties including rhizosphere or intercellular colonization, plant growth promotion, increase of plant nutrient availability through nitrogen fixation or phosphate solubilization, potential to degrade aromatic compound and ability to form symbiotic interactions with plants (Suárez-Moreno et al., 2010). For example, a strain of *Burkholderia tropica*, isolated from tomato rhizosphere in Mexico, have shown mineral phosphate-dissolving capacity, which has allowed this strain to be defined as a plant growth promoting rhizobacterium (Caballero-Mellado et al., 2007).

The *Burkholderia cepacia* complex (Bcc), a group of closely related *Burkholderia* species, contains different strains that include human and plant pathogens to biocontrol agents. Human pathogens have represented a major concern about the use of this complex
as biocontrol; nowadays, the use of *Burkholderia cepacia* complex has been banned (Parke, 2000).

The genus *Mitsuaria* was discovered in 1999 when a strain formerly known as *Matsuebacter chitosanotabidus* (Park et al., 1999) was isolated from soils from Matsue, Japan with the purpose of isolating chitosanase-producing bacteria. Lately, a new name was proposed by Park and Amakata’s group who named it as *Mitsuaria chitosanitabida* (Amakata et al., 2005). Little is known about this genus besides its chitinolytic capacities and the potential antagonistic activity of its chitinolytic enzymes against phytopathogenic fungi and ability to grow in the rhizosphere (cabbage, soybean and petunia among others) (Someya et al., 2011).

In 2008, Benitez and McSpadden Gardener isolated two strains belonging to the two genres *Mitsuaria* and *Burkholderia* from soils identified as disease suppressive of soilborne diseases (Baysal, F. et al. 2008). Isolation started with a population-based approach to correlate the presence of microbial populations with the disease suppression using Terminal Restriction Fragment Length Polymorphism (T-RFLP). *Mitsuaria* and *Burkholderia* strains were marked by different TRFs revealed positive association with disease suppression (Benitez, 2008). When screening these isolates, *Mitsuaria* sp. strain H24L5A showed clear distinction from the known *M. chitosanitabida* with 98 to 99% of strain identity according to the Basic Local Alignment Search Tool (BLAST). Moreover, the *Burkholderia* sp. strain showed also clear distinction from *Burkholderia cepacia* with only 96% identity. Several isolates of both genres were tested for their biocontrol capacity by conducting a pathogen growth inhibition test *in vitro* and *in vivo* against *Pythium aphanidermatum, Phytophthora capsici, Pythium sylvaticum, Phytophthora*
sojae, Rhizoctonia solani, Fusarium graminearum, Alternaria solani and Fusarium oxysporum. Mitsuaria sp. isolates showed great capacity for *in vitro* inhibition regardless of the pathogen. Moreover, they all showed chitinolytic activity when *tested in vitro*. On the other hand, *Burkholderia* sp. isolates showed variable, less frequent inhibition and no chitinolytic activity in the in-vitro assays. For in-vivo assays with tomato and soybean seedlings, H24L5A Mitsuaria sp. strain reduced lesion severity on soybean by 15% and by 20% on tomato seedlings compared to a negative control. Moreover, *Burkholderia* sp. isolates also lead to lesion reduction (Benitez and McSpadden Gardener, 2009). In further work with Mitsuaria sp. strain H24L5A, Rong et al. (2012) obtained the draft genome sequences. This study revealed H24L5A DNA sequence identity of 53% and 53.7% with Leptothrix cholodnii SP-6 and Methylibium petroleiphilum PM1. In addition, some of the protein-encoding genes not shared with these species were identified principally as genes encoding chitinase, chitosinase and cellulases, which is consistent with the positive chitinolytic activity showed *in vitro* (Benitez and McSpadden Gardener, 2009; Rong et al., 2012).

The biocontrol mechanism used by the strains described above can be one or more of the following: nutrient competition, iron competition, antibiotic production and secretion of lytic enzymes, and inducing resistance in the plant (Gnanamanickam, 2002). The mechanism described last is important at the moment of elucidating biocontrol activity of the agents applied, especially the ones that are never in contact with the pathogen. In fact, induced resistance can act at distance and can provide systemic protection. Local infection by bacterial, viral or fungal strains can induce the synthesis of signal molecules that can spread systemically throughout the plant leading to induced
systemic resistance (ISR). ISR is relatively easy to demonstrate with rhizobacteria acting as biocontrol agents that survive on the roots but still can protect the plants against foliar pathogens (Gnanamanickam, 2002). A local oxidative burst, followed by a hypersensitive response, which is characterized by programmed cell death and necrosis in the site infection, are characteristics of ISR (Heil, 2001). There are some signals that should be triggered, first, the rhizobacterial strain should produce ISR elicitors and the plant as well should have a matching receptor and an inducible defense pathway downstream that can activate the system after recognizing it. Reports of microbial mediated ISR on diverse crops like cucumber, bean, radish, tobacco and tomato against fungi, bacteria, virus and insects have been published (Gnanamanickam, 2002).

Biological control has also been part of integrated pest management (IPM). IPM can be defined as a system in which hosts, pathogens, environment and socioeconomic components interact. Tomato is intensively managed and requires high economic input, and therefore, there is a strong need to fulfill economic and environmental requirements through effective pest control methods. Indeed, the use of rhizobacteria in an intensive management program should be considered, taking into account factors like nitrogen fertilization and mulch as well as fungicide spray programs in order to optimize the management of tomato diseases (Nava Diaz, 2005).

According to most updated information, in 2009 total tomato production (fresh marked and processing) in Ohio reached a yield of 23 tonnes per acre, which paces it in the fifth place in tomato production among the states (United States Department of Agriculture, 2010). Due to the importance of this crop and the high economic impact that diseases may have on it, it important to provide supporting evidence of disease control
that may help to overcome this outbreaks. Bacterial speck caused by *Pseudomonas syringae* pv. tomato and bacterial spot caused by a set of species in the genus *Xanthomonas* are among the most devastating diseases of tomato. These strains are classified into four pathogen groups as separate species: *X. euvesicatoria* (group A), *X. vesicatoria* (group B), *X. perforans* (group C), and *X. (group D)*, where strains belonging to groups A, B and D infect both tomato and pepper and Group C strains infect only tomato (Jones et al., 2004; Potnis et al., 2011). *X. gardneri* has especial importance in Ohio due to the highly warm and moist summer conditions that can favor disease development resulting in dramatic crop losses. Indeed, a recent report of its presence in the territories of Michigan and Ohio was published by Ma et al (2011).

Moreover, according to the USDA, the United States produces about 13% of the world’s wheat and supplies about 25% of the world’s wheat export market (USDA, 2012). Ohio is the leading producer of soft red winter wheat. Soft red winter wheat is planted in the fall and harvested in the early summer. It has excellent milling and baking characteristics, it is also used to make flour and as an improvement for blending. In addition, hard red spring wheat has the highest protein content and is used to make hearth breads (Ohio Corn and Wheat Growers Association, 2011). Two diseases are of special concern on wheat in Ohio: *Septoria tritici* blotch and *Stagonospora nodorum* blotch, the latter considered a major disease worldwide with reduced yield consequences in wheat (Lipps and Mills, 2002). Indeed, *Stagonospora nodorum* is a foliar pathogen that became an important pathogen in the Midwest by the late 1980s. Management of *Stagonospora* leaf blotch can be achieved through cultural practices like crop rotation, fungicide seed treatments, application of foliar fungicides, resistant cultivars and biological control.
(Engle et al., 2006). This last has been explored by the use of non-host pathogens as biocontrol agents. For example, Nolan and Cooke (2000) inoculated wheat flag leaves with *Drechslera teres*, a barley pathogen, and obtained significant reduction of disease severity and significant increase in yield (Nolan and Cooke, 2000).

The main goal of this work was to test the biocontrol and plant growth promotion capacity of *Mitsuaria* sp. strain H24L5A and *Burkholderia* sp. strain R4F2. Both bacterial strains showed *in vitro* biocontrol capacity against fungal plant pathogens on initial experiments performed in 2009 and it was hypothesized that both strains would control *X. gardneri* infections in tomato and *S. nodorum* infections in wheat. In addition, it was hypothesized that pre-commercial and commercial products that showed plant growth promotion activity would also have activity against plant pathogens.

**MATERIALS AND METHODS**

*Plant material, pathogens and microbial inoculants*

Hybrid tomato *Solanum lycopersicum* ‘Celebrity F1’ (Johnny’s Selected Seed, Winslow, ME) and organic ‘Hard Red RB07’ spring wheat (*Triticum aestivum*) (Albert Lea Seed House, Albert Lea, MN) seeds were used in the bioassays. Seeds of tomato were hot water-treated (Miller and Lewis 2005), air dried, packaged in clean plastic Petri dishes and stored in the cold room before being sown (10°C). *Xanthomonas gardneri* strain SM230-10, causal agent of bacterial leaf spot was provided by Dr. Sally Miller laboratory (Wooster, OH) (Ma et al, 2011) and *Stagonospora nodorum* strain, was provided by Dr. Pierce Paul laboratory (Wooster, OH). The microbial inoculants tested in this study, divided into three groups with a description of each treatment are described in
Table 3.1; in addition, tap water was applied as a negative control (NC). Bacterial cultures were maintained in King’s broth B (DIFCO Laboratories, Detroit, MI) in 30% glycerol at -80°C, fresh cultures were prepared from the frozen stock on 1/10 Tryptic Soy Agar plates (DIFCO Laboratories, Detroit, MI). Bacterial suspensions were prepared on 1/10 Tryptic Soy broth. The Algae-Based Biofertilizer (ABB) was prepared from a frozen stock on BG-11 Agar (Atlas, 1995); cell suspensions were prepared on BG-11 liquid medium (Atlas, 1995).

Biocontrol and plant growth bioassays under greenhouse conditions

Seeds were sown in an inorganic, chemically and stable growing medium composed of 60% industrial grade 20:40 silica sand (Best Sand, Chardon, OH) and 40% (w:w) Primera One calcinated clay field conditioner. Tomato seeds were sown initially on seedling trays previously filled with growing medium, afterwards, when seedlings developed their first two true leaves, each seedling was transplanted into single plastic cone-tainers of 164 ml volume, 3.8 cm diameter and 21 cm of depth (Stuewe & Sons, Inc., Tangent, OR) set up in trays. Single wheat seeds were sown directly into each cone-tainer. In addition, 50 ml of a modified Hoagland’s solution consisting of reagent grade 0.5 mol/L KCl, 0.5 mol/L K$_2$SO$_4$, 0.5 mol/L MgSO$_4$·7H$_2$O, 0.05 mol/L Ca(H$_2$PO$_4$)$_2$·2H$_2$O, 0.01 mol/L CaSO$_4$·2H$_2$O and 0.5 mol/L KH$_2$PO$_4$ along with a solution of 10 ppm of NH$_4$NO$_3$ diluted in distilled water were added weekly to each plant (Bumgarner et al., 2012).

Biocontrol and plant growth promotion were tested in two different set of experiments in the greenhouse, each consisting of three experiments of tomato and two of
wheat, all performed under greenhouse conditions with a day/night range of temperature of 10-25°C monitored by data loggers (B-Series Watch Dog, Spectrum Technologies Inc, IL). Each cone-tainer was watered with 50 ml of water daily. A randomized complete block design per experiment, consisting of nineteen treatments with five replicates each, was used as experimental design. Microbial inoculants were applied once in the plant growth promotion experiments and twice in the biocontrol experiments by using a soil-drenching method, consisting on pouring 50ml of each treatment into the corresponding cone-tainer. For the biocontrol experiments, the first application of treatments for tomato was at transplanting and two weeks after sowing for wheat; the second application was eight weeks after transplanting for tomato and at the eight week after sowing the wheat seeds. Plant growth promotion experiments were carried out with the first application described above. In the tomato biocontrol bioassays, X. gardneri strain SM230 was sprayed at a final concentration of 1.55 X 10^6 cells/ml to one single leaf per seedling as a cell suspension in 1/3 King’s broth B (DIFCO Laboratories, Detroit, MI). Challenged seedlings were about 3-5 inches tall and had 2-6 true leaves developed. All inoculated leaves were covered with plastic bags for 18 hours.

After incubation time, bags were removed and challenged leaves were tagged with a wire. Symptom development of bacterial spot (chlorotic haloes, yellowing as well as curly leaves and presence of small brown lesions) was monitored daily and disease severity was recorded 7 days after pathogen inoculation using a disease severity scale described in Figure 3.1.

In the wheat biocontrol assays, S. nodorum was sprayed to the entire wheat plants at a concentration of 1.59 X 10^6 spores/ml; afterwards, plants were covered with plastic
bags for 24 hours. Leaf blotch symptoms (i.e. small chlorotic lesions on the leaves ranging from yellow-brown to red-brown) were monitored after 7 days and disease severity was recorded after 12 days of pathogen inoculation using a 0 to 4 rating scale as follows: 0 = healthy plant, 1 = up to 20% of the plant showing chlorotic lesions, 2 = 25% up to 50% of the plant showing chlorotic lesions, 3 = 50% up to 75% of the plant showing chlorotic lesions, 4 = 75% up to 100% of the plant showing chlorotic lesions. Biocontrol and plant growth promotion experiments in tomato ended nine weeks after transplanting and seven weeks after planting in wheat. In both sets of experiments plants’ height, shoot biomass, root length and root biomass were assessed. In the case of wheat, presence of tillers and heads and number of leaves in the plant growth bioassay were also recorded.

Biocontrol and plant growth bioassays under field conditions

Bacterial cell suspensions in 1/10 Tryptic Soy Broth (DIFCO Laboratories, Detroit, MI) codified as MIE1 S and MIE2 S as well as 1X solutions of the experimental 3, formulated pre-commercial and commercial microbial inoculants described in Table 3.1 and water as negative control (NC) were tested under field conditions in tomato. Tomato seedlings were sown in organic potting soil (Better-Gro Pot-n-gro, Better-Gro LTD, Millersburg, OH), placed in an organic greenhouse and watered daily until they reached V2 growth stage; seedlings then were transplanted into the field. Treatments were applied by soil-drenching at a volume of 150 ml per plant at transplanting and a second application took place two weeks after transplanting. The field bioassay consisted of a randomized complete block design with eight treatments in a plot composed by eight raised beds or blocks of 96 ft each with a distance of 10 feet among beds. Each treatment
was applied to 8 individual plants separated from each other by 1.5 feet. Irrigation tape was put along all the beds, covered with plastic mulch. Watering was scheduled for 4 hours three times a week. After five days of the second application of treatments, a single leaf of one plant (4-6 fully extended leaves) per treatment corresponding to each block was sprayed with *X. gardneri* strain SM230 at a concentration of $1.55 \times 10^6$ cells/ml. Leaves were covered with plastic bags for 18 hours, then challenged leaves were tagged with a wire. Symptom development of bacterial spot (chlorotic haloes, yellowing as well as curly leaves and presence of small brown lesions) was monitored daily and disease severity was recorded 7 days after pathogen inoculation as percentage of diseased leaf that ranged from 0 to 100%. Plant growth evaluation corresponding to the first sampling was carried out by recording plants’ height and shoot biomass of one plant per treatment corresponding to each block. A second and final evaluation consisted on recording plants height of five plants per treatment per block and shoot biomass of one plant per treatment corresponding to each block.

*Statistical analysis*

Analyses of variance were run on MINITAB® (v16.1.0 Minitab Inc., State College, PA) using a General Linear Model and pairwise comparisons of means were performed based on Tukey’s Honestly Significant Difference (HSD) test. To analyze the association of tiller and heads presence with the application of treatments, a Chi-squared test was carried out.
RESULTS

Biocontrol effects of microbial inoculants in greenhouse bioassays in tomato

Significant evidence of microbial inoculants’ effects on biocontrol in tomato was found at $P<0.05$. The most significant reduction in leaf spot severity was 39% on average provided by the application of MIE1 RC (50%-43%-22%) compared to the negative control (NC). Percentages in the parenthesis presented in this and the following sections of results correspond to each of the three individual experiments carried out in tomato or each of the two individual experiments carried out in wheat (data not shown).

In addition, although not statistically different, disease severity was reduced in average by 20-29% by the application of the following treatments: MIE1 S (39%-26%-13%), MIE2 RC (33%-20%-13%), MIE2 S (11%-20%-22%), MIE2 SM (22%-31%-32%) and MIC1 3X (33%-15%-20%) when compared to the negative control (Table 3.2).

There was significant evidence of the effects of microbial inoculants on plant growth promotion when assessing shoot height, shoot weight and root weight ($P<0.05$). Shoot height and shoot weight were increased by 25% and 64% respectively on average followed by the application of MIE1 SM (34%-36%-13% for height and 118%-108%-40% for weight) compared to the negative control ($P<0.05$). Shoot height and shoot weight were increased by 20% by the application of MIE1 (13%-29%-20% for height and 47%-76%-38% for weight) in average respectively, compared to the negative control ($P<0.05$) (Table 3.2).
Biocontrol effects of microbial inoculants in greenhouse bioassays in wheat

No significant evidence of microbial inoculants’ effect on reduction of leaf blotch severity in wheat was found \((P>0.05)\). However, a tendency of low values of disease severity corresponding to 26\% of disease reduction was seen followed by the application of MIC1 1X \((33\% - 22\%)\) compared to the negative control (NC) (Table 3.3).

On the other hand, significant evidence of the effects of microbial inoculants on plant growth promotion was found when assessing shoot weight and root weight \((P<0.05)\). Shoot weight was increased significantly in average by 85\% followed by the application of MIE2 S \((100\%-54\%)\) and MIE2 SM \((76\%-55\%)\) compared to the NC \((P<0.05)\). Also, root weight was increased by up to 70\% followed by the application of MIE2 S \((70\%-75\%)\) and MIE2 SM \((35\%-89\%)\) compared to the NC \((P<0.05)\) and shoot weight was increased significantly in average by 85\% followed by the application of MIE1 SM \((117\%-37\%)\) \((P<0.05)\). Although not statistically different, shoot weight and root weight were increased in average by 20-40\% followed by the application of MIE1 S \((47\%-36\% \text{ for shoot weight and } 25\%-18\% \text{ for root weight})\) compared to the negative controls (Table 3.3).

Plant growth promotion effects of microbial inoculants in greenhouse bioassays in tomato

Significant evidence of microbial inoculants’ effects on plant growth promotion in tomato was found when assessing shoot height, shoot weight and root weight \((P<0.05)\). Shoot height was significantly increased by 25-28\% and shoot weight by 53-59\% in average followed by the application of MIE2 S \((45\%-35\%-19\% \text{ for height and } 76\%-89\%\)
45% for shoot weight) and MIE2 SM (54%-20%-20% for height and 100%-82%-26% for shoot weight) \( (P<0.05) \). Additionally, shoot weight was also significantly increased by 45% followed by the application of MIE1 SM (76%-74%-26%) when compared to the negative control \( (P<0.05) \). Root weight was increased in average by 25% followed by the application of MIE2 S (18%-64%-17%) compared to the NC \( (P<0.05) \) (Table 3.4).

*Plant growth promotion effects of microbial inoculants in greenhouse bioassays in wheat*

No significant evidence of microbial inoculants’ effects on plant growth promotion in wheat was found when assessing shoot height, shoot weight, root length and root weight \( (P>0.05) \). However, there was a tendency towards higher values of root weight with an increase in average of 24-36% followed by the application of MIE1 S (17%-32%), MIE2 S (60%-20%) and MIE2 SM (33%-38%) compared to the negative control. No evidence of association \( (P>0.05) \) between presence of heads and tillers and application of treatments was found (Table 3.5).

*Biocontrol and plant growth promotion effects of microbial inoculants in field bioassays in tomato*

No significant evidence of microbial inoculants’ effects on plant growth promotion and disease severity reduction was found in the first sampling \( (P>0.05) \). However, there was a trend towards lower values of disease severity followed by the application of MIE1 S, which reduced disease severity by 12% compared to the negative control (Table 3.6). In addition, there was a tendency towards increase in shoot height by 20% followed by the application of MIE2 S. Another trend towards increase in shoot
weight was followed by the application of the following treatments: MIE3 1X, MIE2 S, MIF2 1X, MIC2 1X, MIE1 S and MIF1 1X, which corresponded to 54%, 52%, 46%, 44%, 40% and 22% of shoot weight increase (Table 3.6).

In addition, no significant evidence of microbial inoculants’ effects on plant growth promotion was found in the second sampling ($P>0.05$). However, there was a trend towards increase in shoot weight by 10% followed by the application of MIE2 S and MIC2 1X. In addition there was a trend towards increase in tomato yield by 4% followed by the application of MIE1 S and MIE2 S (Table 3.7).

**Temperature Measurements**

Temperature patterns inside the greenhouse fluctuated between a minimum 12°C and a maximum of 44°C. Temperatures reported for wheat bioassays ($A^Y$ and $B^Y$) in Figure 3.2, reached a maximum of 44°C and a minimum of 12°C. Temperatures reported for tomato bioassays ($C^Y$, $D^Y$ and $E^Y$) in Figure 3.2, reached a maximum of 44°C and a minimum of 17°C.

**DISCUSSION**

Biocontrol capacity of *Mitsuaria* sp. strain H24L5A and *Burkholderia* sp. strain R4F2 was first suggested by Benítez et al. (2007), who associated markers for *Mitsuaria* and *Burkholderia* with plant disease suppression in field studies of a transitional organic system (Benítez et al., 2007; Baysal et al., 2008.). Strain H24L5A showed in-vitro and seedling inhibition of fungal and oomycete pathogens inoculated in tomato and soybeans.
In this study, the application of H24L5A and R4F2 strains led to bacterial leaf spot severity reduction in tomato. H24L5A activity was attributed to re-suspended cells and cell suspensions, providing up to 39% of bacterial leaf spot reduction, and R4F2 activity to re-suspended cells, cell suspensions and spent medium, providing up to 29% of disease reduction (Table 3.2). Actinovate®, a commercial microbial inoculant provided up to 25% bacterial leaf spot reduction, which suggests that the experimental bacterial strains could provide greater disease reduction. In addition, the application of R4F2 and H24L5A as cell suspensions and spent medium increased tomato and wheat growth by 20% to 64% (Table 3.4 and Table 3.5). This suggests that the presence of H24L5A cells in the treatments is important for biocontrol and also suggests the synergistic effect of H24L5A and R4F2 applied with 1/10 Tryptic Soy Broth (DIFCO Laboratories, IL) on growth of tomato and wheat.

Biocontrol capacity of H24L5A in tomato may be attributed to the expression of genes encoding chitinase, chitosanase and cellulases, which have been found in its genome sequence (Rong et al., 2012). Previously, in vitro data suggested that biocontrol by Mitsuaria strain H24L5A could be derived from its chitinolytic capacity as well (Benitez and McSpadden Gardener, 2009); however, such activities are not known to lead to induced resistance in the absence of soilborne fungi, whose cell walls may be degraded. As described by Eckardt (2008), plant resistance could be triggered by a number of surface-derived molecules, which can elicit a general immune response. Chitinases naturally present on Mitsuaria strain H24L5A and the ones produced by plants as a result of plant defenses activation, could accumulate at the site of inoculation accompanied by rests of cell walls (composed primarily by cellulose), which can function
as elicitors of downstream defense response genes and can result in induced systemic resistance (ISR) in plants against numerous pathogens (Eckardt, 2008; Nandakumar et al., 2001). H24L5A as well as the rest of the treatments were drenched into the tomato roots and they never found to contact each other, thus protective effect could be plant-mediated and could be attributed to induced systemic resistance (van Peer et al. 1991, van Loon and Bakker, 2005; Chincholkar and Mukerji, 2007). In addition, the trends found in our field results provide information to only speculate that H24L5A could reduce bacterial leaf spot severity under field conditions as well (Table 3.6).

Biocontrol capacity of R4F2 may be attributed to the presence of a Bral/R AHL-like quorum sensing system found in diazotrophic Burkholderia, which has been proposed to be present in all species of the plant-associated cluster of Burkholderia (Suarez-Moreno et al. 2008). This system regulates exopolysaccharides (EPS) production, which may resemble fungal elicitors that activate plant defenses by the perception of plant receptors present in the root cells (Gnanamanickam, 2002). EPS could also make colonization efficient through the formation of biofilms, which could also protect and act as a sink for the nutrients in the rhizosphere, reducing the availability of nutrients from the root exudates for local pathogen colonization (Haggag, 2010).

H24L5A and R4F2 plant growth promotion capacity could be attributed to nitrogen fixing capacities, aromatic compound degradation and phosphate solubilization (Suarez-Moreno et al., 2008; Haggag, 2010; Xu, 2012).

Both bacterial cell suspension and spent medium promoted tomato and wheat growth. Both treatments were composed by 1/10 Tryptic Soy Broth (DIFCO Laboratories, IL). In the case of R4F2, the application of cell suspensions led to greater
values in the parameters of plant growth when compared to the spent media, suggesting that the presence of cells is important for plant growth promotion. However, the concentrations of N, P and K in 1/10 Tryptic, which were 0.17 g/l, 0.3 g/l and 0.12 g/l respectively, exceeded the concentrations of N, P and K corresponding to 1.4 mg/l, 0.21 mg/l and 0.26 mg/l in Actinovate® (Ramsdell and Whittier, 1944; Harmon et al., 1969; Crawford and Suh, 1995). This suggests that H24L5A and R4F2 formulations with 1/10 TSB had more nutrients available for microbial survival, which could have been taken up by the bacterial agents and by the microbes present in the rhizosphere; moreover, part of it could have also been taken up by the plants. The solution of 1/10 Tryptic Soy Broth suspension functioned as a carrier that provided a food base to aid proliferation of the bacterial cells that could have acted as biofertilizers. In fact, according to Burges (1998), nutrition of bacterial cells applied to plants affects their plant growth promotion capacity by allowing the production of certain enzymes that can trigger plant growth.

Hoagland’s solution with N, P and K concentrations of 0.01 g/l, 3.1 g/l and 13 g/l respectively, provided the plants with 10% less nitrogen than 1/10 Tryptic Soy Broth, which suggesting that plant growth enhancement may have occurred partly due to chemical factors. Spent medium could contain secondary metabolites secreted by the bacterial cells that could act as enhancers of plant growth such as: enzymes, signaling molecules like autoinducers that could regulate biofilm formation of natural populations of bacteria, and plant growth regulators like gibberellins, cytokinins and indole acetic acid (reviewed by Nandakumar et al., 2001; Teasdale et al., 2009).

Given the trends, we can only speculate that the application of ABB could exceed and/or match the values on plant growth led by the application of Re-Start™ in the
greenhouse and field bioassays. This speculation suggests that consortia interactions with the plant could lead to growth enhancement. As ABB is a new experimental product, little is known about its mechanisms to promote plant’s health, but there is evidence of plant biomass enhancement on lettuce, tomato, turf and wheat under different conditions suggesting its plant growth promotion capacity (S. Park and B. McSpadden Gardener, unpublished).

Moreover, we also speculate that the application of Abundance™ could exceed and/or match the values on plant growth led by the application of Actinovate®. This speculation suggests that Abundance™ interactions with the plant could lead to plant growth enhancement due to the biological and chemical components of the powdered formulation. Kaolin clay, as a enhancer on the performance the dry bacterial cells contained in the formulation, which could have helped in the induction of plants’ growth increase, kaolin as a protection barrier, dried yucca extract as a sticky agent which could have helped in the adherence of the formulation to the roots and yeast extract, as a source of nutrients. In wheat, given the trends, we can only speculate that the application of Actinovate® could have reduced the values of leaf blotch. This results suggest that Actinovate ® 1X could reduce disease severity in the conditions established in this study. As the pathogen was sprayed in the whole plant, the active ingredient of Actinovate ® 1X, S. lydicus could have triggered plant defense responses after colonization by the excretion of lytic enzymes such as chitinase and cellulose (Fernandez et al, 2011).

Variation in biocontrol responses on wheat and tomato could be due to the inherent differences between monocots and dicots defense response signals. The signal transduction molecules involved in the resistance mechanisms in dicot plants may differ
from those in monocot plants. For example, in dicot plants, jasmonic acid (JA) was suggested to mediate induced resistance; in monocots, both salicylic acid (SA) and jasmonic acid induced resistance have been found. However, knowledge of induced systemic resistance mechanisms in monocots is limited, due to the fact that few reports have demonstrated that the effective colonization of roots of any monocot species by beneficial microorganisms can induce systemic disease resistance to leaf pathogens (reviewed by Djonović et al., 2007). Evidence of resistance responses triggered by defense-related signal compounds such as SA or chemical analogs have been described in monocots demonstrating low or no efficacy. For example, Gorlach et al. (1996) studied the application of benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) in a field bioassay in wheat to control of septoria leaf blotch, but did not find any evidence of control (reviewed by Vallad and Goodman, 2004).

In addition, difference in the crop responses to the microbial inoculants applications could also be attributed to abiotic stress due to the variable temperature at the greenhouse. Temperatures inside the greenhouse ranged from 12°C to 44 °C (Figure 3.2). The optimum temperature for spring wheat is 25° and the maximum is 30°C-32°C; and the optimum temperature for tomato is 30°C and the maximum is 35°C (Gould, 1983; Khan and Shewry, 2009). A variance of spatial and temporal thermal attributes in the environment can cause stress in plants, as a result, changes on plants’ physiology and metabolism take place. In this study, high peaks of temperature showing values exceeding the maximum for plants’ growth were recorded for both crops. However, daily temperature alteration, could have influence wheat leaves elongation rate and growth. According to Friend (1965) wheat growth under optimal temperatures led to an increase
in leaf and tiller rate, but exceeding maximum temperatures leads to reduced leaf numbers and total tiller numbers. When plants are exposed to low and high temperature stress, various adaptive, protective and deleterious responses can occur (Friend, 1965). In the case on wheat, morphological adaptations leading to a controlled growth could have taken place, as high temperature stress particularly could reduce yield of wheat. A mechanism of heat tolerance is the synthesis of proteins called heat shock proteins, which accumulate in cells and help alleviate heat stress. Differentiation in tomato growth could have been due to heat shock proteins aggregate into a granular structure in the cytoplasm, possibly protecting the whole machinery of protein synthesis. This is a phenomenon not detected in wheat, which makes it more susceptible to heat stress when compared to tomato (Bazzaz et al, 1996; Hopkins and Hüner, 2004; Neumann et al., 2008).
<table>
<thead>
<tr>
<th>Microbial inoculant</th>
<th>Type</th>
<th>Description</th>
<th>Rate</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mitsuaria</em> sp. strain H24L5A</td>
<td>Experimental 1</td>
<td>Betaproteobacteria isolated from mixed hay disease suppressive soil applied as cell suspension on 1/10 Tryptic Soy Broth</td>
<td>$10^7$ cells/ml</td>
<td>MIE1 S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell re-suspension in water obtained after centrifuging cell suspension on 1/10 Tryptic Soy Broth</td>
<td>~$10^7$ cells/ml</td>
<td>MIE1 RC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spent medium which corresponds to the supernatant of the centrifuging process performed to separate cells</td>
<td></td>
<td>MIE1 SM</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp. strain R4F2</td>
<td>Experimental 2</td>
<td>Betaproteobacteria isolated from mixed hay disease suppressive soil applied as cell suspension on 1/10 Tryptic Soy Broth</td>
<td>$10^7$ cells/ml</td>
<td>MIE2 S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell suspension in water obtained after centrifuging cell suspension on 1/10 Tryptic Soy Broth</td>
<td>~$10^7$ cells/ml</td>
<td>MIE2 RC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spent medium which corresponds to the supernatant of the centrifuging process performed to separate the cells</td>
<td></td>
<td>MIE2 SM</td>
</tr>
<tr>
<td><em>Algae</em>-Based Biofertilizer (ABB)</td>
<td>Experimental 3</td>
<td>Mixed culture composed of green algae and bacteria, isolated from the surface of a low carbon, sand-based growth medium. Green biofilm grown on liquid medium and applied as liquid suspension</td>
<td>$10^6$ CFU/ml</td>
<td>MIE3 1X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>~$10^5$ CFU/ml</td>
<td></td>
<td>MIE3 ½X</td>
</tr>
</tbody>
</table>

Continued

Table 3.1. Microbial inoculants, prepared as different treatments, applied in tomato and wheat greenhouse and field bioassays to determine their effects on biocontrol and plant growth.
<table>
<thead>
<tr>
<th>Microbial inoculant</th>
<th>Type</th>
<th>Description</th>
<th>Rate</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinovate ®</td>
<td>Commercial 1</td>
<td>Commercial biofungicide, active ingredient: <em>Streptomyces lydicus</em> strain WEYC 108, isolated from the rhizosphere of linseed plant roots. Powder mix diluted with water</td>
<td>2.1 g/L</td>
<td>MIC1 3X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.7 g/L</td>
<td>MIC1 1X</td>
</tr>
<tr>
<td>Re-Start ™</td>
<td>Commercial 2</td>
<td><em>Ecklonia maxima</em> cytoplasm concentrate. Liquid product diluted with water</td>
<td>5 ml/L</td>
<td>MIC2 1X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.5 ml/L</td>
<td>MIC2 ½X</td>
</tr>
<tr>
<td>AZO</td>
<td>Formulated pre-commercial 1</td>
<td><em>Azospirillum</em> sp. powder formulation. Dried cells diluted with water</td>
<td>1.4 g/L</td>
<td>MIF1 2X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.7 g/L</td>
<td>MIF1 1X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.35 g/L</td>
<td>MIF1 ½X</td>
</tr>
<tr>
<td>Abundance™</td>
<td>Formulated pre-commercial 2</td>
<td><em>Pseudomonas</em> sp. and <em>Bacillus</em> sp. powder formulation. Powder mix composed of kaolin clay, dried plant extract, lime, and freeze-dried bacteria. diluted with water</td>
<td>5.1 g/L</td>
<td>MIF2 1X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.55 g/L</td>
<td>MIF2 ½X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.28 g/L</td>
<td>MIF2 ¼X</td>
</tr>
<tr>
<td>Treatment</td>
<td>Disease Severity&lt;sup&gt;y&lt;/sup&gt; (0-5)</td>
<td>Shoot Height&lt;sup&gt;y&lt;/sup&gt; (cm)</td>
<td>Shoot Weight&lt;sup&gt;y&lt;/sup&gt; (g)</td>
<td>Root Length&lt;sup&gt;y&lt;/sup&gt; (cm)</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------</td>
<td>-----------------------------</td>
<td>----------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>MIC1 1X</td>
<td>3.3 abc</td>
<td>10.1 e</td>
<td>2.3 f</td>
<td>21.5 a</td>
</tr>
<tr>
<td>MIC1 3X</td>
<td>2.9 abc</td>
<td>10.9 abcde</td>
<td>2.7 def</td>
<td>21.3 a</td>
</tr>
<tr>
<td>MIC2 ½X</td>
<td>3.6 ab</td>
<td>10.2 cde</td>
<td>2.4 ef</td>
<td>22.6 a</td>
</tr>
<tr>
<td>MIC2 1X</td>
<td>3.9 a</td>
<td>10.9 bcde</td>
<td>2.7 ef</td>
<td>22.6 a</td>
</tr>
<tr>
<td>MIE1 RC</td>
<td>2.3 c</td>
<td>9.7 e</td>
<td>2.8 def</td>
<td>23.1 a</td>
</tr>
<tr>
<td>MIE1 S</td>
<td>2.8 abc</td>
<td>13.1 abc</td>
<td>4.4 abc</td>
<td>21.6 a</td>
</tr>
<tr>
<td>MIE1 SM</td>
<td>3.7 ab</td>
<td>13.8 a</td>
<td>5.1 a</td>
<td>21.7 a</td>
</tr>
<tr>
<td>MIE2 RC</td>
<td>2.9 abc</td>
<td>10.5 bcde</td>
<td>3.0 def</td>
<td>21.2 a</td>
</tr>
<tr>
<td>MIE2 S</td>
<td>3.1 abc</td>
<td>13.3 ab</td>
<td>4.5 ab</td>
<td>22.5 a</td>
</tr>
<tr>
<td>MIE2 SM</td>
<td>2.7 bc</td>
<td>13.0 abcd</td>
<td>4.0 abcd</td>
<td>21.9 a</td>
</tr>
<tr>
<td>MIE3 ½X</td>
<td>3.4 abc</td>
<td>10.0 e</td>
<td>2.9 def</td>
<td>22.7 a</td>
</tr>
<tr>
<td>MIE3 1X</td>
<td>3.2 abc</td>
<td>10.0 cde</td>
<td>2.4 ef</td>
<td>22.1 a</td>
</tr>
<tr>
<td>MIF1 ½X</td>
<td>3.5 abc</td>
<td>10.8 abcde</td>
<td>2.8 def</td>
<td>22.6 a</td>
</tr>
<tr>
<td>MIF1 1X</td>
<td>3.6 ab</td>
<td>10.9 abcde</td>
<td>2.7 def</td>
<td>22.3 a</td>
</tr>
<tr>
<td>MIF1 2X</td>
<td>3.8 ab</td>
<td>11.0 abcde</td>
<td>2.5 ef</td>
<td>23.4 a</td>
</tr>
<tr>
<td>MIF2 ¼X</td>
<td>3.3 abc</td>
<td>10.5 bcde</td>
<td>2.8 def</td>
<td>22.9 a</td>
</tr>
<tr>
<td>MIF2 ½X</td>
<td>4.0 a</td>
<td>12.2 abcde</td>
<td>3.5 bcde</td>
<td>22.3 a</td>
</tr>
<tr>
<td>MIF2 1X</td>
<td>3.5 abc</td>
<td>11.4 abcde</td>
<td>3.1 cdef</td>
<td>22.3 a</td>
</tr>
<tr>
<td>NC</td>
<td>3.8 ab</td>
<td>11.0 de</td>
<td>3.1 ef</td>
<td>21.3 a</td>
</tr>
</tbody>
</table>

Table 3.2. Effect of microbial inoculants on disease severity, shoot height, shoot weight, root length and root weight of tomato plants infected with *X. gardneri* in greenhouse experiments.

<sup>y</sup> Values correspond to the means of three experiments with five replicates each. Tukey’s multiple comparison results are presented as a grouping table, treatments that share a letter are not significantly different.

<sup>z</sup> *P*-values correspond to the ANOVA tables at an alpha value set at 0.05.
Table 3.3. Effect of microbial inoculants on disease severity, shoot height, shoot weight, root length and root weight of wheat plants infected with *S. nodorum* in greenhouse experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease Severity(^y) (0-5)</th>
<th>Shoot Height(^y) (cm)</th>
<th>Shoot Weight(^y) (g)</th>
<th>Root Length(^y) (cm)</th>
<th>Root Weight(^y) (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC1 1X</td>
<td>1.9 b</td>
<td>34.5 a</td>
<td>1.0 c</td>
<td>21.0 a</td>
<td>2.0 c</td>
</tr>
<tr>
<td>MIC1 3X</td>
<td>2.3 ab</td>
<td>36.8 a</td>
<td>1.1 c</td>
<td>21.7 a</td>
<td>2.0 c</td>
</tr>
<tr>
<td>MIC2 (\frac{1}{2}X)</td>
<td>2.3 ab</td>
<td>35.3 a</td>
<td>1.1 c</td>
<td>24.2 a</td>
<td>1.9 c</td>
</tr>
<tr>
<td>MIC2 1X</td>
<td>2.4 ab</td>
<td>39.1 a</td>
<td>1.1 c</td>
<td>22.8 a</td>
<td>2.3 bc</td>
</tr>
<tr>
<td>MIE1 RC</td>
<td>2.6 ab</td>
<td>36.1 a</td>
<td>1.4 bc</td>
<td>23.1 a</td>
<td>3.1 abc</td>
</tr>
<tr>
<td>MIE1 S</td>
<td>2.2 ab</td>
<td>39.7 a</td>
<td>2.0 abc</td>
<td>21.4 a</td>
<td>2.9 abc</td>
</tr>
<tr>
<td>MIE1 SM</td>
<td>2.4 ab</td>
<td>37.5 a</td>
<td>2.6 a</td>
<td>20.5 a</td>
<td>3.4 abc</td>
</tr>
<tr>
<td>MIE2 RC</td>
<td>2.6 ab</td>
<td>36.4 a</td>
<td>1.0 c</td>
<td>21.8 a</td>
<td>1.9 c</td>
</tr>
<tr>
<td>MIE2 S</td>
<td>2.4 ab</td>
<td>38.7 a</td>
<td>2.6 a</td>
<td>22.3 a</td>
<td>4.1 a</td>
</tr>
<tr>
<td>MIE2 SM</td>
<td>2.7 ab</td>
<td>37.3 a</td>
<td>2.4 ab</td>
<td>23.1 a</td>
<td>4.0 ab</td>
</tr>
<tr>
<td>MIE3 (\frac{1}{2}X)</td>
<td>2.8 ab</td>
<td>38.3 a</td>
<td>1.0 c</td>
<td>22.7 a</td>
<td>2.3 bc</td>
</tr>
<tr>
<td>MIE3 1X</td>
<td>2.2 ab</td>
<td>36.5 a</td>
<td>1.1 c</td>
<td>22.3 a</td>
<td>2.2 c</td>
</tr>
<tr>
<td>MIF1 (\frac{1}{2}X)</td>
<td>2.8 ab</td>
<td>38.3 a</td>
<td>1.1 c</td>
<td>23.7 a</td>
<td>2.5 abc</td>
</tr>
<tr>
<td>MIF1 1X</td>
<td>2.6 ab</td>
<td>37.7 a</td>
<td>1.2 c</td>
<td>23.7 a</td>
<td>2.5 abc</td>
</tr>
<tr>
<td>MIF1 2X</td>
<td>2.5 ab</td>
<td>34.8 a</td>
<td>0.9 c</td>
<td>22.6 a</td>
<td>2.3 bc</td>
</tr>
<tr>
<td>MIF2 (\frac{1}{4}X)</td>
<td>2.3 ab</td>
<td>37.3 a</td>
<td>1.1 c</td>
<td>22.6 a</td>
<td>2.2 c</td>
</tr>
<tr>
<td>MIF2 (\frac{1}{2}X)</td>
<td>3.0 a</td>
<td>37.4 a</td>
<td>1.4 bc</td>
<td>22.5 a</td>
<td>2.9 abc</td>
</tr>
<tr>
<td>MIF2 1X</td>
<td>2.4 ab</td>
<td>37.8 a</td>
<td>1.4 bc</td>
<td>22.5 a</td>
<td>2.4 abc</td>
</tr>
<tr>
<td>NC</td>
<td>2.6 ab</td>
<td>36.8 a</td>
<td>1.4 c</td>
<td>21.5 a</td>
<td>2.4 c</td>
</tr>
</tbody>
</table>

\(^y\) Values correspond to the means of two experiments with five replicates each. Tukey’s multiple comparison results are presented as a grouping table, treatments that share a letter are not significantly different.

\(^z\) P-values correspond to the ANOVA tables at an alpha value set at 0.05.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot Height (cm)</th>
<th>Shoot Weight (g)</th>
<th>Root Length (cm)</th>
<th>Root Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC1 1X</td>
<td>13.1 cd</td>
<td>3.7 cd</td>
<td>21.7 a</td>
<td>2.9 b</td>
</tr>
<tr>
<td>MIC1 3X</td>
<td>12.4 cd</td>
<td>3.4 cd</td>
<td>23.2 a</td>
<td>3.0 ab</td>
</tr>
<tr>
<td>MIC2 (\frac{1}{2})X</td>
<td>13.3 bcd</td>
<td>3.6 cd</td>
<td>24.7 a</td>
<td>3.3 ab</td>
</tr>
<tr>
<td>MIC2 1X</td>
<td>13.2 cd</td>
<td>3.3 d</td>
<td>21.6 a</td>
<td>3.0 b</td>
</tr>
<tr>
<td>MIE1 RC</td>
<td>12.7 cd</td>
<td>3.5 cd</td>
<td>23.2 a</td>
<td>2.8 b</td>
</tr>
<tr>
<td>MIE1 S</td>
<td>15.2 abc</td>
<td>4.8 abc</td>
<td>21.3 a</td>
<td>3.5 ab</td>
</tr>
<tr>
<td>MIE1 SM</td>
<td>15.2 abc</td>
<td>5.4 ab</td>
<td>22.2 a</td>
<td>3.7 ab</td>
</tr>
<tr>
<td>MIE2 RC</td>
<td>13.4 bcd</td>
<td>3.8 cd</td>
<td>24.5 a</td>
<td>3.3 ab</td>
</tr>
<tr>
<td>MIE2 S</td>
<td>16.5 a</td>
<td>5.9 a</td>
<td>21.8 a</td>
<td>4.0 a</td>
</tr>
<tr>
<td>MIE2 SM</td>
<td>16.2 ab</td>
<td>5.7 a</td>
<td>22.2 a</td>
<td>3.7 ab</td>
</tr>
<tr>
<td>MIE3 (\frac{1}{2})X</td>
<td>12.5 cd</td>
<td>4.1 bcd</td>
<td>21.7 a</td>
<td>3.6 ab</td>
</tr>
<tr>
<td>MIE3 1X</td>
<td>12.2 cd</td>
<td>3.3 cd</td>
<td>24.1 a</td>
<td>3.1 ab</td>
</tr>
<tr>
<td>MIF1 (\frac{1}{2})X</td>
<td>13.7 abcd</td>
<td>4.1 bcd</td>
<td>22.3 a</td>
<td>3.1 ab</td>
</tr>
<tr>
<td>MIF1 1X</td>
<td>13.8 abcd</td>
<td>3.8 cd</td>
<td>24.8 a</td>
<td>3.0 ab</td>
</tr>
<tr>
<td>MIF1 2X</td>
<td>13.2 bcd</td>
<td>4.0 bcd</td>
<td>23.2 a</td>
<td>3.3 ab</td>
</tr>
<tr>
<td>MIF2 (\frac{1}{4})X</td>
<td>13.5 abcd</td>
<td>3.8 cd</td>
<td>24.4 a</td>
<td>3.2 ab</td>
</tr>
<tr>
<td>MIF2 (\frac{1}{2})X</td>
<td>13.4 bcd</td>
<td>3.9 cd</td>
<td>23.0 a</td>
<td>3.1 ab</td>
</tr>
<tr>
<td>MIF2 1X</td>
<td>13.1 bcd</td>
<td>4.0 bcd</td>
<td>23.3 a</td>
<td>3.3 ab</td>
</tr>
<tr>
<td>NC</td>
<td>12.9 d</td>
<td>3.7 d</td>
<td>23.0 a</td>
<td>3.2 b</td>
</tr>
</tbody>
</table>

| P-value\(z\) | <0.05 | <0.05 | 0.12 | <0.05 |

Table 3.4. Effect of microbial inoculants on shoot height, shoot weight, root length and root weight of tomato in greenhouse experiments.

\(y\) Values are the means of three experiments with five replicates each. Tukey’s multiple comparison results are presented as a grouping table, treatments that share a letter are not significantly different.

\(z\) P-values correspond to the ANOVA tables at an alpha value set at 0.05.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot</th>
<th>Root</th>
<th>Number of leaves</th>
<th>Head presence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Height (cm)</td>
<td>Weight (g)</td>
<td>Length (cm)</td>
<td>Weight (g)</td>
</tr>
<tr>
<td>MIC1 1X</td>
<td>41.5 a</td>
<td>1.5 a</td>
<td>22.7 a</td>
<td>2.9 a</td>
</tr>
<tr>
<td>MIC1 3X</td>
<td>40.1 a</td>
<td>1.8 a</td>
<td>23.1 a</td>
<td>3.1 a</td>
</tr>
<tr>
<td>MIC2 ½X</td>
<td>40.0 a</td>
<td>1.5 a</td>
<td>22.7 a</td>
<td>2.4 a</td>
</tr>
<tr>
<td>MIC2 1X</td>
<td>42.6 a</td>
<td>1.8 a</td>
<td>22.4 a</td>
<td>2.5 a</td>
</tr>
<tr>
<td>MIE1 RC</td>
<td>40.9 a</td>
<td>1.8 a</td>
<td>21.5 a</td>
<td>2.5 a</td>
</tr>
<tr>
<td>MIE1 S</td>
<td>41.6 a</td>
<td>1.9 a</td>
<td>22.9 a</td>
<td>3.1 a</td>
</tr>
<tr>
<td>MIE1 SM</td>
<td>41.5 a</td>
<td>1.7 a</td>
<td>23.3 a</td>
<td>3.4 a</td>
</tr>
<tr>
<td>MIE2 RC</td>
<td>40.3 a</td>
<td>1.5 a</td>
<td>23.4 a</td>
<td>2.4 a</td>
</tr>
<tr>
<td>MIE2 S</td>
<td>43.2 a</td>
<td>1.9 a</td>
<td>22.3 a</td>
<td>3.3 a</td>
</tr>
<tr>
<td>MIE2 SM</td>
<td>44.0 a</td>
<td>2.1 a</td>
<td>23.1 a</td>
<td>3.3 a</td>
</tr>
<tr>
<td>MIE3 ½X</td>
<td>40.6 a</td>
<td>1.5 a</td>
<td>22.8 a</td>
<td>2.4 a</td>
</tr>
<tr>
<td>MIE3 1X</td>
<td>40.7 a</td>
<td>1.7 a</td>
<td>22.3 a</td>
<td>2.4 a</td>
</tr>
<tr>
<td>MIF1 ½X</td>
<td>41.8 a</td>
<td>1.7 a</td>
<td>21.3 a</td>
<td>2.7 a</td>
</tr>
<tr>
<td>MIF1 1X</td>
<td>40.5 a</td>
<td>1.6 a</td>
<td>22.0 a</td>
<td>2.7 a</td>
</tr>
<tr>
<td>MIF1 2X</td>
<td>41.5 a</td>
<td>1.6 a</td>
<td>23.1 a</td>
<td>2.4 a</td>
</tr>
<tr>
<td>MIF2 ½X</td>
<td>41.4 a</td>
<td>1.6 a</td>
<td>23.3 a</td>
<td>2.2 a</td>
</tr>
<tr>
<td>MIF2 1X</td>
<td>39.5 a</td>
<td>1.5 a</td>
<td>22.1 a</td>
<td>2.5 a</td>
</tr>
<tr>
<td>NC</td>
<td>41.2 a</td>
<td>1.6 a</td>
<td>22.5 a</td>
<td>2.5 a</td>
</tr>
</tbody>
</table>

| P-value  | 0.865 | 0.254 | 0.860 | 0.556 | 0.18 | 0.30 | 0.53 |

Table 3.5. Effect of microbial inoculants on shoot height, shoot weight, root length, root weight, number of leaves and presence of tillers and heads of wheat in greenhouse experiments.

* Values correspond to the means of two experiments with five replicates each. Tukey’s multiple comparison results are presented as a grouping table, treatments that share a letter are not significantly different.

* Values correspond to the frequencies of two experiments with five replicates each with a total of 10 per each treatment except for NC where a total count was 45.

* P-values correspond to the ANOVA tables at an alpha value set at 0.05 for all the variables except for tillers and heads presence, where a chi-square test was applied.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease Severity(^y) (0-5)</th>
<th>Shoot Height(^y) (cm)</th>
<th>Shoot Weight(^y) (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC1 1X</td>
<td>56.2  (a)</td>
<td>29.1  (a)</td>
<td>166.3  (a)</td>
</tr>
<tr>
<td>MIC2 1X</td>
<td>61.2  (a)</td>
<td>35.7  (a)</td>
<td>292.1  (a)</td>
</tr>
<tr>
<td>MIE1 S</td>
<td>45.0  (a)</td>
<td>35.0  (a)</td>
<td>282.1  (a)</td>
</tr>
<tr>
<td>MIE2 S</td>
<td>51.3  (a)</td>
<td>37.4  (a)</td>
<td>308.0  (a)</td>
</tr>
<tr>
<td>MIE3 1X</td>
<td>48.8  (a)</td>
<td>33.5  (a)</td>
<td>310.4  (a)</td>
</tr>
<tr>
<td>MIF1 1X</td>
<td>57.5  (a)</td>
<td>33.3  (a)</td>
<td>247.1  (a)</td>
</tr>
<tr>
<td>MIF2 1X</td>
<td>57.5  (a)</td>
<td>32.6  (a)</td>
<td>295.2  (a)</td>
</tr>
<tr>
<td>NC</td>
<td>51.2  (a)</td>
<td>31.0  (a)</td>
<td>201.7  (a)</td>
</tr>
</tbody>
</table>

\(P\)-value\(^z\) 0.527 0.163 0.124

Table 3.6. Effect of microbial inoculants on disease severity, shoot height, and shoot weight of tomato corresponding to the first sampling of a field experiment carried out in June 2012.

\(^y\) Values are the means of one plant belonging to each treatment per eight replicates. Tukey’s multiple comparison results are presented as a grouping table, treatments that share a letter are not significantly different.

\(^z\) \(P\)-values correspond to the ANOVA tables at an alpha value set at 0.05.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot Height&lt;br&gt; (cm)</th>
<th>Shoot Weight&lt;br&gt; (kg)</th>
<th>Tomato yield/plant&lt;br&gt; (kg)</th>
<th>Tomatoes per plant&lt;br&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC1 1X</td>
<td>66.0 a</td>
<td>1.8 a</td>
<td>2.4 a</td>
<td>34.8 a</td>
</tr>
<tr>
<td>MIC2 1X</td>
<td>69.2 a</td>
<td>2.1 a</td>
<td>2.6 a</td>
<td>39.9 a</td>
</tr>
<tr>
<td>MIE1 S</td>
<td>66.3 a</td>
<td>1.8 a</td>
<td>2.7 a</td>
<td>38.6 a</td>
</tr>
<tr>
<td>MIE2 S</td>
<td>66.4 a</td>
<td>2.1 a</td>
<td>2.7 a</td>
<td>34.1 a</td>
</tr>
<tr>
<td>MIE3 1X</td>
<td>65.5 a</td>
<td>1.6 a</td>
<td>2.3 a</td>
<td>34.3 a</td>
</tr>
<tr>
<td>MIF1 1X</td>
<td>63.8 a</td>
<td>1.9 a</td>
<td>2.4 a</td>
<td>29.1 a</td>
</tr>
<tr>
<td>MIF2 1X</td>
<td>65.3 a</td>
<td>1.8 a</td>
<td>2.2 a</td>
<td>30.4 a</td>
</tr>
<tr>
<td>NC</td>
<td>69.1 a</td>
<td>1.9 a</td>
<td>2.6 a</td>
<td>38.9 a</td>
</tr>
</tbody>
</table>

| P-value<sup>z</sup> | 0.15 | 0.80 | 0.96 | 0.64 |

Table 3.7. Effect of microbial inoculants on shoot height, shoot weight, tomato yield per plant and tomatoes per plant corresponding to the second sampling of a field experiment carried out in August 2012.

<sup>x</sup> Values are the means of five plants belonging to each treatment per eight replicates. Tukey’s multiple comparison results are presented as a grouping table, treatments that share a letter are not significantly different.

<sup>y</sup> Values are the means of one plant belonging to each treatment per eight replicates. Tukey’s multiple comparison results are presented as a grouping table, treatments that share a letter are not significantly different.

<sup>z</sup> P-values correspond to the ANOVA tables at an alpha value set at 0.05.
Figure 3.1. Severity of symptoms of bacterial leaf spot in tomato using a 0 to 5 rating scale as follows: 0 = healthy leaf, 1 = 20% of the leaf shows symptoms, 2 = 40% of the leaf shows symptoms, 3 = 60% of the leaf shows symptoms, 4 = 80% of the leaf shows symptoms, and 5 = the complete leaf presents yellowing and starts to curl.

Figure 3.2. Daily maximum and minimum temperatures (°C) recorded in the greenhouse during the development of the bioassays with tomato and wheat.

\textsuperscript{3}Bioassays carried out in wheat

\textsuperscript{2} Bioassays carried out in tomato
CHAPTER 4

Challenges and future perspectives for microbial biocontrol agents

The first attempts in biocontrol of plant pathogens took place between 1920 and 1940 when C. Hartley introduced antagonistic fungi to control a plant pathogen that caused damping-off in pine seedlings sown in treated soil. Disease reduction when inoculating the antagonists on sterile soil was first attributed to the competition among different microbial communities in the soil (Cook and Baker, 1983). Until now, this capacity is also attributed to single isolates applied in sufficient concentrations (Kim et al., 2011). Microbial diversity has led to the discovery of various biocontrol agents based in their natural capacity to provide biocontrol. Indeed, when testing fungal and bacterial isolates from the environment for biocontrol activities, among 1% to 10% show some antagonistic capacity against pathogens in vitro (McSpadden Gardener and Fravel, 2002).

To assess the activity of microbial biocontrol agents on in vivo systems, initial in vitro experiments are usually done. Even though this will not guarantee their effectiveness on in vivo experiments due to the multiple factors involved (i.e. medium composition, light, temperature and pH), it will provide a starting point for further research. Greenhouse testing represents the first step to test the isolates on in vivo environments. Controlled conditions may provide enough approximation to the field.
environment depending on how bioassays are performed. However, once taken to the field, microbial biocontrol agents can act differently from the way they did in the greenhouse. This is primarily due to the interaction with other microbial communities that can only occur naturally in the field and can somehow influence their activity (Gnanamanickam, 2002).

After biocontrol activity has been proved on *in vivo* testing (i.e. greenhouse and field experiments), improving their efficacy is the next step. One approach could be trying different formulations until finding the most accurate for the biocontrol agent. To find out the suitability of the microbial biocontrol agents as an alternative to control plant diseases, it is imperative to know their status on the market and on the research and development area. Reliable data as well as confirmed protocols are needed for isolation and preparation of biocontrol products. A marketing background, as well as deep knowledge about the registration process will help novel researchers to overcome the challenge of placing biocontrol agents as a reliable alternative for growers.

Screening, production, registration and distribution of biocontrol agents are all the processes involved in the application biocontrol in agriculture. These processes represent the links in a chain, which will remain strong only if each one is monitored and developed correctly.

*Field testing, a challenge to microbial biocontrol agents*

Microbial biocontrol agents are live organisms that need to overcome a series of obstacles to survive and proliferate in the matrix they have been inoculated. Is in the
transition from laboratory to the field where the capacity of overcoming these obstacles is tested to the maximum level. This challenge has been difficult even for successfully registered biocontrol agents due to the heterogeneity of the inhabitants of the soil and the lack of successful protocols to scale up the laboratory production. Knowing more about the ecology and micro-ecology surrounding biocontrol agents can facilitate optimization of their efficacy. Trying to describe the membership of the community by making an inventory of the rhizosphere or phyllosphere communities can help to understand associations between the inhabitants. General studies of community dynamics depending on time and environment could reveal decisive data for biocontrol agents’ survival and performance. Palazzini et al (2007), used the dominance index (I_D) to compare competitive ability of microbial species under particular environmental conditions. This is practically an in-vitro assay where the interactions among the pathogen and the isolates are scored under adjusted conditions like water activity and temperature. This research determined in-vitro interactions under conditions that simulate those occurring during flowering (Palazzini et al., 2007). This approach gives an alternative over dual cultures and gives a realistic approximation of natural conditions and represents an alternative to other in vitro assays. Moreover, it opens the alternative to manipulate the medium, as it is known that lack of correlation between in vitro and in plant assays may be due to the influence of the test medium.

Nutrient status, which represents the availability of food for the biocontrol agents, also contributes to their efficacy on the field (Gnanamanickam, 2002; Fravel, 2005). An approach to know more about this is the use of biosensors to provide nutritional
information about the biological control agents. For example, studies on *Pseudomonas fluorescens* strain A506 determined the direct relationship of antibiotic production against fire blight with iron availability (Temple et al., 2004). Information like this can provide data that will help to predict biocontrol efficacy of biocontrol agents.

Community signaling represents another key factor in biocontrol. One approach is to assess the signals related to the mechanism of quorum sensing. Quorum sensing plays a central role in bacterial gene regulatory networks and consists in controlling gene expression depending on a determined concentration of bacterial population with the production of signal molecules. This system is involved in processes like conjugation, symbiosis and virulence, triggering systemic resistance and biofilm formation in bacteria. Further analysis targeting the relationship between quorum sensing and regulatory systems related to biocontrol capacity like the system GacS-GacA need to be assessed. This will provide information about signal cascades and regulatory networks that may occur in plant-microbe interactions (Wei and Zhang, 2006; Liu et al., 2011).

*Scaling up and formulation microbial biocontrol products*

Developing effective methods for commercial scale systems is another challenge in the production of microbial biocontrol agents (Canamas et al., 2008). To obtain semi-commercial amounts of the biocontrol agent, in order to be applied at field scales, it is necessary to scale up the production process, at least to a pilot-plant level (Patiño-Vera et al., 2005). In terms of production, cells need to be tolerant to transportation and storage;
the medium, besides providing enough nutrients, should be produced at low-cost and at large quantities.

There is not enough research published about scaling-up aspects of fermentation of biocontrol agents. Patiño-Vera et al. (2005), conducted a pilot-scale production for *Rhodotorula minuta* yeast, a biocontrol agent, in which a 100 L pilot fermentor was developed. He and also tested two different mediums in order to find the most suitable. As a result, an alternative medium with low cost was found for the mass production of the biocontrol agent and viable cells were produced at a large scale (Patiño-Vera et al., 2005).

Developing methods to manipulate biocontrol agents’ cells is also an approach that has been taken into account at the time of scaling up processes. For example, controlling resistance of microorganisms to temperature stress by following a shift in the incubation treatment has proved to be an alternative to yeast cells. In 2008, Canamas et al. induced thermo tolerance to yeast cells by mild heat treatments, allowing the cells to survive higher temperatures when being processed by spray drying for further formulation. However, even though cells survival was achieved, viability was not enough to consider spray drying as dehydration method for commercialization (Canamas et al., 2008). Thus, more research conducted towards improving cells’ survival against environmental factors when scaling up production processes is needed.

From a technical standpoint, effective formulations require previous knowledge about the biology of the biocontrol organism, the pathogen, environment, and community dynamics as described above. Indeed, being live organisms has profound impact in the
manufacturing processes of biocontrol agents. The ability to survive and replicate could increases the need of formulation depending on the target and the stage of the biocontrol agent (Burges, 1998). Finding the correct formulation for commercial products containing biocontrol agents is not easy. Time and money need to be invested to find the correct amendment, sometimes with unsuccessful results. Most of the commercialized products have gone through a wide range of transitions over time to finally find a suitable formulation. For example, products containing *B. thuringiensis* have undergone a broad variety of formulation changes, which started form aqueous suspensions and wettable powders to dry flowable granules (Schisler et al., 2004). Development of a commercially-viable formulation will require a complex understanding of common application practices and equipment. Ingredients must be safe and acceptable to regulatory agencies in all areas; there have been situations where formulations could have damaged the crop. To avoid this, all of the ingredients used must be tested first to make sure the crop is not being harmed (Leggett et al., 2011). There are no specific approaches for formulation of biocontrol products as every species/strain differs from each other. Variation could be present even within the same species, which make it necessary to develop separate research and development processes for each organism, which could raise the cost of production. In fact, sometimes the products that are already in the market do not represent the best possible formulation due to limitations in cost (Burges, 1998).

Continuous research improvements in formulations can help to achieve the goal of successful incorporation of biocontrol products into agriculture production. Therefore, formulations represent a key component at the time of determining biocontrol agents’
efficacy as well as other processes involved in discovery, production and stabilization of the biomass. If the biocontrol agent strains do not reach the stage of formulation on a stable manner, the commercial potential of this agent will be compromised (Schisler et al., 2004).

*Storage and distribution of commercial microbial biocontrol agents*

In general, after the biocontrol agent is produced, formulated and packaged, it is stored in the producer’s warehouse until being shipped to retailers. This is the first storage room, which is under charge of the producer, who can adjust the storage environment to optimum conditions to maintain the original concentration of the cells. However, when the product leaves the warehouse, it has to deal with different environmental conditions that may affect its stability. It is important to define the most essential storage factors that may limit the efficacy of the product. Durability in store has had priority on research and new approaches like the use of new granule carriers or the addition of preservatives are being the focus of new research that will possibly lead to less costly technologies with greater efficacy. Moreover, it is at this point where packaging plays an important role providing the product limiting exposure to environmental changes. Several kinds of packaging have been developed, most of them depending on the final composition of the product. For example, for liquid suspensions, plastic and hermetic containers have been used and for powdered formulations, bags provided with reinforced structures to ensure resistance to handling have been used (Fravel, 2005; Leggett et al., 2011).
Transferring the technology of microbial inoculants to the growers

Researchers, specifically plant pathologists, play an important role at the moment of transferring technology to the growers. They could design field bioassays to test the biocontrol agents alone or in combination with other products like pesticides, which will provide information about the integration of biocontrol in the production system, leading to integrated programs for disease control. This work needs to be continuous as growers get used to the use of biocontrol agents as alternatives for plant disease management (Fravel, 2005). In production, experience of growers and farmers is important as it and provides information about their conventional agricultural practices. With this regard, regional difference is also important, for example in Canada growers use peat-based and seed- applied inoculants while in the US growers prefer liquid inoculants (Ehlers, 2011).

Moreover, in order to reach the growers’ interest, it is important to take into account their needs and experiences. Basically the challenge is to fulfill their requirements of low cost, easiness at the moment of prepare and apply as well as good performance. Easiness and performance can always be improved by altering formulation to make it friendlier to the user (Burges, 1998).

Cost can also be improved by taking care of the formulation, storage and delivery ways. For example, preparing concentrate biomass in liquid formulations is practical and can also minimize costs of transportation but is highly dependable on the temperature of storage, which implies that product should be stored and distributed under refrigerated conditions (Abadias et al., 2003). This may not represent an obstacle but a challenge that needs to be overcome by more friendly ways of storage and distribution that can fit the
growers’ and farmers’ practices. Therefore, more studies related to understand how farmers and growers react to the use of biocontrol agents need to be done. This will allow researchers and industry to design better and efficient techniques of production, application and distribution, as well as national and local governments to design efficient policies for their use (Moser et al., 2008). For example, in 2008, Moser et al. developed the first comparative study to know farmer’s perceptions about biocontrol as part of an integrated pest management of strawberry on three different regions. As a result, they found factors that influenced most growers’ confidence on biocontrol: personal hands-on experience, positive publicity and advertising. Thus, this research should represent the starting point of further research related to this topic, allowing researchers and industry personnel to know more about farmers’ perception.

Status of biocontrol agents in the market

According to marketing statistics, the global percentage of biopesticides has been growing since 1997 and keeps growing at a rate of 10% per year (Bailey, Boyetchko and Längle, 2010). In 2007, global biopesticide marked reached $512 million representing about 2.4% of the overall pesticides market. Interestingly, it is expected to reach more than $1 billion by 2012, which represents about 4.2% of the overall market. USA, Canada and Mexico use about 44% of all biocontrol products sold worldwide, about 53 microbial biopesticides are registered in the US. The European Union has registered 21 microbial biopesticides, accounting for 20% of the global biopesticide market. The remaining is divided by 20% for the Oceanic countries, 10% for Latin America countries and about
5% to Asia and India (Marrone, 2007; Bailey et al., 2010; Chandler et al., 2011). An analysis of the market is imperative to know the size of the target market and avoid the drop-outs of products due to the unwillingness of agrochemical companies to undertake the development for small markets (Burges, 1998). This data provide useful information about the global status of biopesticides and the increased demand worldwide.

**Regulatory systems for microbial pesticides**

According to The Environmental Protection Agency (EPA), microbial pesticides are part of what we know as biopesticides, defined as mass-produced agents manufactured from a living microorganism or a natural product and sold for the control of plant pests (Chandler et al., 2011; EPA, 2012). Due to the fact that they represent microbial products sold for control of plant diseases, they must be registered with the Environmental Protection Agency (EPA) in the US. EPA regulates the use of pesticides in general under the authority of three federal statutes: Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Federal Food, Drug, and Cosmetic Act (FFDCA), and the Food Quality Protection Act (FQPA). EPA also regulates three classes of biopesticides: biochemical pesticides, microbial pesticides, and plant-incorporated protectants (PIPs). The principal aims of regulation are to ensure that their use will not cause unreasonable adverse effects to human health or the environment and to characterize the products that are provided by the manufacturers to ensure their good quality and reliability (Fravel, 2005; EPA 2010; Chandler et al., 2011). Regarding to human safety, test to assess the pathogenicity, toxicity, and allergenicity of biopesticides are demanded (Fravel, 2005;
Moreover, this control is getting more and more necessary that other countries are considering including regulatory systems for biocontrol agents. The challenge now is to make sure that when this is done, it is carried out in a realistic and flexible way with the only purpose to inform and warn about their safety (Blum et al., 2003; Chandler et al., 2011). The decision to authorize or not the use of a biopesticide for commercial purposes needs to be made based on expert opinion within a regulatory plan. It is important that the personnel involved in that plan have expertise with biopesticides. In fact, professionals like plant pathologists, entomologists or environmentalist are required to get involved in this process due to their competency in this area. In fact, when regulators lack expertise within the biopesticides area, they could tend to delay making a decision or ask for information that sometimes goes beyond what it is needed (Chandler et al., 2011).

*Future perspectives for microbial biocontrol agents*

Due to the growing demand on environmentally friendly products and the aim to help growers to reduce the use of chemical pesticides, biocontrol research has been growing in the last decade. The increasing demand for organic products and the concern on sustainability in the agricultural system will contribute to the development of new strategies that could for disease control. One approach to this challenge is to develop a total system to pest management in which the farms are made resistant to pests and therapeutic treatments are provided as a second line for defense. First, crops will have to be managed in a way that naturally pest regulators systems could provide biocontrol and
secondly by the use of plants bred with defenses against pests. Inside this system, integrated pest management would work by promoting natural pest regulation and environmentally friendly technologies (Speiser et al., 2006; Chandler et al., 2011). Join effort from the government, researchers and industry needs to be focused towards increase education of users about pesticides. Specifically, companies will need to invest in educating their costumers and positioning of their biocontrol products in the marketplace through experienced partners. Government should increase federal funding towards the development of efficacy trials and demo programs and researchers should increase applied research integrating biopesticides and chemicals (Marrone, 2007). Moreover, developing new microbial biopesticides will raise the necessity of teams composed by: marketing people, who can understand the needs of the farmers; agronomists and plant pathologists, who can understand plant-pathogen interactions; microbiologists, who can understand the physiology of the biocontrol agent; chemists, who can understand the physical/chemical properties of formulations, and statisticians that can design and analyze experiments, which will ensure accurate approaches as well as efficient methodologies that will position microbial biopesticides in the market (Bailey et al., 2010).
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