Studies on Re-colonization of Microbial Populations in the Rhizosphere of Chrysanthemum after Soil Steaming

by

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Abstract

Among soil disinfestation practices, the application of steam to the soil prior to cultivation is one of the most commonly used management strategies. Growers use steam to effectively kill pathogens by heating the soil. Recently, growers of cut-flowers have noticed a reduction in flower size and weight, which they attribute to steaming the soil. Since shorter and lighter plants lose their commercial value, and therefore, are not accepted in the international market, there is a need to study in more detail the effects of steaming soil on plants and their microbial communities. The overall objective of this study was to determine the effect of consecutive steam application on the rhizosphere soil microbial community of chrysanthemum, and in particular the effects on specific bacterial groups (*Pseudomonas* spp. and *Bacillus* spp.). We hypothesized that repeated steam disinfestations cause irreversible changes in the soil community structure, leading to increases of bacterial groups that can be allelopathic for the plant, and that applications of plant growth-promoting rhizobacteria (PGPR) will remediate those effects. To examine the effects of steaming on the rhizosphere microbiota a greenhouse pot assay experiment was conducted using chrysanthemum cultivar golden Kent as a model plant. Rhizosphere soil samples were collected from chrysanthemum plants cultivated in soil that was subjected to steam once, steamed once with PGPR, steamed twice, and not steamed. Our results indicated that repeated steaming of soil changed the abundance of the microbial community, in particular increasing *Pseudomonas* populations. Although, the characterization of the functional traits of *Pseudomonas* cultured isolates showed that they produced differential levels of IAA and HCN, steaming the soil did not result in changes in functional traits of the *Pseudomonas* species

compared to isolates from non-steamed soil. Therefore, the magnitude of the effect of soil steaming is related more to the abundance and composition of the pseudomonad community than the functional traits of any individual *Pseudomonas* species. Additionally, PGPR did not cause changes in the abundance of fluorescent pseudomonads but did have effects on rhizosphere and endophyte colonization. To assess the effect of consecutive steaming on the soil bacterial communities, DGGE, a molecular technique based on the 16S rDNA was used. Results indicated that steaming the soil once does not have a major impact on the soil eubacterial community structure of the rhizosphere, but does impact to some extent the soil microbial community when reapplied. However, potentially important differences in the communities of specific bacterial groups (*Pseudomonas* and *Bacillus)* are caused by steaming. There are many ways in which steaming the soil can affect the microbial properties of the soil. We found that steaming the soil increases the abundance of one of the main rhizosphere groups, the fluorescent pseudomonads, and that those changes are related to chrysanthemum growth reduction in the field.

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List of Abbreviations

AEFB	Aerobic spore forming bacteria
CFU	Colony forming units
CRD	Completely randomized design
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DRB	Deleterious rhizobacteria
FDS	Fern distortion Syndrome
HCN	Hydrogen cyanide
HR	Hypersensitive reaction
IAA	Indole acetic acid
PCR	Polymerase chain reaction
PGPR	Plant growth promoting rhizobacteria
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SE	Standard error
USD	United States dollars

Chapter I. Studies on re-colonization of microbial populations in the rhizosphere of chrysanthemum after soil steaming

Introduction

Commercial floriculture is a very important sector of agriculture in both developing and developed countries. In Holland and Colombia, for example, flower production is a dominant industry and flower exports generate important revenue. In the United States, the floral and nursery crop industry is one of the fastest growing agriculture sectors, and in 2006 cut flower sales alone generated $411 million USD (Columbia, 2003).

To satisfy the market demand, flowers are planted repeatedly throughout the year on the same soil at high densities of plants per unit area. In addition to quantity, flower markets also require high quality and blemish-free products. High density and repeated cropping affect soil properties and increase populations of pathogens in the soil, which results in heavy application of pesticides to suppress pest populations (Pizano, 2002).

Management strategies to control soil-borne pathogens and pests of flowers include the use of soil disinfestations (Eshel et al., 2000). Among soil disinfestations, one of the most common management practices is the application of steam to the soil prior to cultivation. Growers use steam to effectively kill pathogens by heating the soil to levels that cause protein coagulation or enzyme inactivation (Klibanov, 1983; Pullman et al., 1981). An advantage of this practice is that it eliminates the need for chemical fumigants that can leave toxic residues in the soil, which results in a need for delayed planting after fumigation while the soil is aerated. Steamed soil, however, is ready for planting immediately after the soil has cooled. Although the

use of steaming as a disinfestation practice is useful for managing plant pathogens and weeds, there is no consensus among scientists about the long-term impact of repetitive use of this practice on the soil microbial community.

Several studies have examined the effects of steam on soil properties and on the re-colonization of the soil by various micro-arthropods. One such study found that during steam disinfestation, levels of manganese increase, and symptoms of manganese toxicity can result, depending on the sensitivity of the crop (Sonneveld and Voogt, 1975). Another study found that edaphic fauna, such as mites and some Coleopteran larvae, can quickly re-colonize the soil and re-establish dense populations 45 days after steam disinfestation (Fenoglio et al., 2006). However, there are no reports examining the long-term effects of steam on indigenous soil microbial communities. It is generally accepted that much of the soil microbial community is eliminated along with the soil-borne pathogens at the time of steam application (Baker, 1962; Chen et al., 1991), but it is not clear how quickly the populations recover and if there are changes in the relative abundance of species within the soil microbial community following steaming. Due to the importance and current use of steam application, it is important to gain an in- depth understanding of the impact on soil microbial communities, including possible long-term effects. The outcome of this study will allow the development of a strategic approach for the frequency of steam application and will determine whether or not changes in soil microbial populations due to steam application will have negative effects on the soil microbial communities and on the crop.

The overall aim of this project is to determine the effect of consecutive steam application on the soil microbial community, in particular on the relative abundance of specific groups (*Pseudomonas* spp. and *Bacillus* spp.). Another aim is to test the hypothesis that repetitive steam

disinfestations cause irreversible changes in the soil community structure, leading to increases of bacterial groups that can be allelopathic for the plant, and lastly to determine the effect of plant growth promoting rhizobacteria (PGPR) applications on microbial communities of steamed soil.

Importance of the cut flower industry

In the United States the total cut flower trade accounts for more than one billion dollars per year at the wholesale level (Jerado, 2006). Two-thirds of total flowers sold in the U.S. are imported from countries such as Colombia, Ecuador, and Holland. Most domestically grown flowers are produced in the Western US (Jerado, 2006). Combining both imported and domestically grown cut flowers, the top selling categories are roses ($1035 million), lilies ($99 million), chrysanthemums ($65 million), *Alstroemeria* ($50 million), carnations ($42 million), tulips ($37 million), and gerbera ($31 million) (Jerado, 2006).

While flower producers are found in both developed and developing countries, the major markets for cut flowers are found in the developed world, with Europe, Japan, and North America as the main importers. Stable climatic conditions and the growth of the world demand for cut flowers make the flower industry an attractive business for developing tropical countries, which take advantage of year-round production and reduced labor cost. Kenya and Colombia, for example, export between 80 and 98 percent of their cut flowers to northern countries (Commandeur and Rooszendaal, 1994).

The increasing demand for imported cut flowers in developed countries makes the phytosanitary regulations at the port of entrance even stricter, to avoid unwanted pathogens and pests. Consequently, one of the primary concerns in flower production is related to plant health management. In addition, high quality standards, such as length and weight of stem, absence of pesticides residues, or any other matter affecting flower appearance, need to be satisfied to

guarantee the marketability of the cut flower. These quality demands come not only from the flower market but also from the consumers. Flowers must be completely free of damage caused by pests or diseases; thus, plant health management is of critical importance. Growers must use a combination of strategies to diagnose, monitor, and treat diseases and pests in a timely manner (Daughtrey and Benson, 2005).

Flower health management

Flowers are grown under the most highly regulated climatic conditions, where temperature, light, and fertilizer regimes are controlled to maximize plant growth. The same conditions, however, are also favorable for pathogens. For instance, warmth and high humidity provide the ideal conditions for foliar growth of pathogens such as *Botrytis* spp. and powdery mildews (Paulitz and Belanger, 2001). In general, flower disease management combines practices such us sanitation, utilization of clean stock and resistant hosts, regulatory actions, and chemical and biological control. Sanitation includes the control of weeds and pests that can be reservoirs for pathogens or vectors. This includes cleanliness of cutting tools during propagation and harvesting of flowers to prevent the spread of pathogens (Khentry et al., 2006), removal of plant debris (Araújo et al., 2005; Daughtrey, 2007), use of soilless media (Lin and Liu, 2007), and water disinfestation (Daughtrey, 2007; Malaure et al., 1991; Zalewska et al., 2007). Examples of host resistance can be found in carnations, where the engineering was able to improve plant resistance to *Fusarium oxysporum f*, one of the causes of major crop losses. There are also reports of resistance in garden roses to powdery mildew by expression of the antimicrobial protein gene (*Ace-AMP1*) (Li et al., 2003; Tanaka et al., 2005).

Flower diseases can also be managed using pesticides, in combination with other practices. The chemical used depends on cost, conditions, and the sustainability of production (Minuto et al.,

1995; Spadaro and Gullino, 2005). Environmental concerns have forced the flower industry to adopt sustainable methods, such as the use of biological control agents for control of pathogens (Whipps, 1997).

Biocontrol agents can be combined with chemical products, physical methods (solarization or steam desinfestation) (Strashnow et al., 1985), or agronomical practices (enhancement of suppressive soils, use of amendments) (Scher and Baker, 1982). Different biocontrol strategies are being developed for different pathogens (Spadaro and Gullino, 2005). For instance, the application of several bacterial strains reduced root colonization by 72-91% and root discoloration by 57-70% in hydroponic chrysanthemums inoculated with *Pythium aphanidermatum* or *Pythium dissotocum.* Among the bacterial strains evaluated, only *Pseudomonas chlororaphis* 63-28 and *Bacillus cereus* HY06 strongly suppressed both *P. aphanidermatum and P. dissotocum* in the chrysanthemum roots, suggesting that bacterial strains can be used to protect chrysanthemums against Pythium root rot (Lin and Liu, 2007; Liu et al., 2007).

Another rhizosphere-competent microorganism, *Trichoderma sp*., has been widely used in flower production. *Trichoderma* spp. possess antifungal systems that consist of a variety of extracellular lytic enzymes that hydrolyze polymers not present in plant tissues (Harman et al., 2004).

Several pathogens have been reported as causal agents of disease in cut flowers. Depending on the flower species and the production system, some diseases will be more severe than others. In chrysanthemum for example, where soil-borne pathogens are very difficult to control, environmentally friendly practices, such as steam disinfestation have proved to be as effective as chemical fumigants (Pizano, 2002). However, some pathogenic or allelopathic

organisms can rapidly re-colonize the soil after steaming, in absence of competition from beneficial groups. In such cases the need for frequency of steaming increases.

Soil disinfestations

Soil disinfestation is defined as the partial reduction of pathogens and microorganisms in the soil (Elsas et al., 1997). Methods of soil disinfestation consist of the use of fumigants (methyl bromide, chloropicrin), irradiation (gamma, ultraviolet, and microwave), or heat (autoclaving, solarization, and steaming) (Ibekwe et al., 2001; Katan and DeVay, 1991; Razavi and Lakzian, 2007). Depending on the flower species and the production system, some methods will be more adequate than others. In general, fumigation and steaming are the most typically used prior to planting of high-value cash crops. In chrysanthemum for example, where soil-borne pathogens are very difficult to control, steam disinfestation has proved to be as effective as chemical fumigants (Pizano, 2002). However, some pathogenic or allelopathic organisms can rapidly re-colonize the soil after steaming, in absence of competition from beneficial groups.

Soil-borne pathogens represent one of the major obstacles in greenhouse production systems. Diseases caused by *Pythium*, *Phytopthora, Rhizoctonia, Fusarium*, and *Sclerotinia* severely impact the flower production industry (Chincholkar and Mukerji, 2007; Daughtrey and Benson, 2005), which is considered a high-value cash crop. Management strategies for these pathogens have involved the use of chemical fumigants, some of which are no longer available (Rosskopf et al., 2005; Roux-Michollet et al., 2008). Considerable effort has been dedicated to looking for new approaches for producing pathogen-free flowers. Among the available approaches, steam disinfestation plays a key role in commercial floriculture for managing diseases and is also a viable alternative to the use of methyl bromide (Klose et al., 2007; Rosskopf et al., 2005; Roux-Michollet et al., 2008).

Baker and Olsen were some of the earliest researchers to work with steaming of soil. They conducted research on pasteurization for the elimination of soil-borne plant pathogens, using moist heat passed through the soil (Baker, 1962; Baker and Olsen, 1960). They used steamed soil in combination with a "retarding organism" (antagonist) to prevent damping off of bedding plants caused by *Rhizoctonia solani*. This steam treatment was designed for selective elimination of pathogens, leaving the resident heat-tolerant micro biota to buffer against any pathogens that might later be introduced into the soil. *Bacillus subtilis* strain A-13 was isolated from Australian soil in a search for antagonism against soil-borne pathogens, and later its growth promotion effects were shown in field studies with cereals. *Bacillus* species were of a particular interest for their survival ability after steam treatment application to the soil. Therefore, after initial screening for growth promotion and antagonism, the final selection was based on the bacteria's ability to survive heat treatment (Cook, 2005).

Currently, there are three methods of disinfesting soil using steam: sheet steaming, negative pressure steaming or **c**ool steaming (Runia 1983). In sheet steaming, the soil is sealed under a thermo resistant sheet before injecting the steam under it. The steam is injected by two parallel pipes placed in trenches between ridges of soil, and each pipe is connected to a valve by which air can be injected through a Venturi inlet (Fenoglio et al., 2006). In the negative pressure steaming, steam is introduced under the steam sheet and pulled into the soil by negative pressures created by a fan. Specifically, the fan draws air out of the soil through buried perforated polypropylene pipes (Runia, 1983). In the cool steaming method, lower temperatures are used (70°C or cool steam). Although steam is considered less drastic an approach than soil fumigants such as methyl bromide and chloropicrin, steam impacts the soil biota as well as physical and chemical properties of the soil (Bollen, 1969; Chen et al., 1991).

Effects of soil disinfestation on biological, physical and, chemical properties of soil

Baker and Olsen (1960) hypothesized that soil disinfestation by steam application would leave some beneficial microorganisms in the soil, rather than creating a biological vacuum. However, recent results have shown that steam disinfestation is not selective and kills most soil biota (Daughtrey and Benson, 2005). Additionally, the high temperatures used to disinfest the soil can regulate soil microbial activity and change bacterial and fungal communities (Bollen, 1969). In general, bacteria are more tolerant to heat than fungi, and further differences in heat tolerance exist within different groups of bacteria and fungi (Hart et al., 2005).

In addition to changes in soil biota, physical and chemical changes may be induced by steaming the soil. Increases in electrical conductivity (Razavi and Lakzian, 2007) as well as nitrogen and manganese levels (Sonneveld, 1979) have been found. In steamed soil the source of nitrogen is the decomposition of soil organic matter and soil biomass. Frequently, steaming results in an increase in NH_4^+-N, and a decrease in NO_3-(Avrahami and Bohannan, 2007). The decrease in NO_3-N has been attributed to leaching of soil and the decrease in the activity of bacteria involved in soil mineralization, leading to accumulation of NH_4^+-N. This indicates that heating causes the release of soluble nutrient minerals from soil organic matter and also from heat-killed biota (Avrahami and Bohannan, 2007; Chen et al., 1991). Manganese is released due to the decomposition of manganese-containing organic compounds. The amount of manganese released will depend on the amount of easily reducible manganese present in the soil, the duration of steaming, and the temperature reached (Sonneveld, 1979). Soil structure and aggregation are physical properties that play an important role in crop production. Some studies have found that disinfesting the soil either by autoclaving (raising the temperature to 120 °C for 20 minutes, waiting 24 hours, and then repeating it) or by using gamma irradiation causes

destruction of soil aggregation to various extents by increasing the soil-clay fraction and decreasing the silt-sized fraction (Berns, 2008). However studies have not yet been conducted to examine the effects of steam disinfestation on soil aggregation.

In summary, steaming of soil can result in drastic changes of microbial biomass, depending on the temperatures reached and the soil moisture (Neary et al., 1999), and it has a direct influence in nitrogen cycling, levels of manganese, and elimination of antagonists. Increases in temperature will not only affect pathogens but also beneficial soil biota (*e.g.*, microbial community composition and density), soil chemical characteristics (*e.g.*, pH, organic matter quantity and quality), as well as soil aggregate stability (Hart et al., 2005; Neary et al., 1999).

Ecological considerations of soil steam disinfestations

Ecologically speaking, the effect of a disturbance on ecosystem stability can be separated into two components: resistance and resilience. Resistance can be defined as the capacity to withstand disturbance, while resilience addresses the capacity to recover after disturbance (Pimm, 1984). Ecosystem resistance and resilience are especially important in agricultural production with a sustainable and environmentally friendly mindset. In the flower production industry, large areas undergo repeated steam treatment with the objective of reducing pathogens, weeds, and pests. The ability of the soil microbial community to recover from this disturbance is crucial for the soil ecosystem because of the role of microorganisms in nutrient cycling processes in soils (Pietikåinen et al., 2005). A comprehensive understanding of the composition of the soil microbial community in high-value cash crops is important in this scenario because steaming the soil might cause changes in functional diversity and consequently select some groups that will compromise the stability and productivity of the agro ecosystem. Experts disagree about the

effects of microbial diversity and microbial community structure on resistance and resilience following disturbance. Some studies have found greater importance in microbial diversity relative to community structure (Girvan et al., 2005), while others have found the opposite (Fierer et al., 2003; Rasmussen and Sørensen, 2001).

Girvan et al, (2005) investigated the potential resilience of soil bacterial communities, the level of genetic diversity, and the relationship between genetic diversity and stability following severe stress due to pollution. For this study two soils that differ in organic matter content were used based on the idea that the higher the organic matter the greater the diversity. Results indicated that the more diverse the soil was the more resistant to pollutants and the higher the resilience after the disturbance. Additionally, functional diversity was not changed (Girvan et al., 2005). The disturbance used by Fierer et al. (2003) consisted of the drying-wetting frequency in two types of soil with inherently different bacterial communities. The effect of this disturbance was only significant in the soil that was not often exposed to changes in moisture. In this soil, shifts in bacterial community composition as well as changes in C mineralization rates were observed. Genetic diversity and richness were not changed relative to drying–rewetting frequency. In the study of Rasmunssen and Sorensen (2001) the effect of mercury contamination (disturbance) on the culturable, functional, and genetic diversity of the bacterial community in soil was investigated. The culturable bacteria and genetic diversity showed an immediate decrease after mercury addition with a recovery by the end of the study. Shift in the community structure was observed and correlated to the recovered diversity. Functional diversity increased right after the disturbance and remained at a level higher than the control. These three studies are examples of how microbial community composition and diversity can change particularly in soils exposed to a significant level of environmental stress.

Although, disinfestation practices are aimed at reducing pathogens and pests in the soil, there are multitudes of communities of nonpathogenic microorganisms that inhabit the soil system as well. Whether or not changes after steaming the soil are related to microbial community structure or diversity remains largely unknown. To answer this, it is logical to first examine the dynamics in microbial communities associated with the rhizosphere of high value crops in which bedding soil has not been steamed, followed by the same analysis after steam application. Duineveld et al. (1998) worked on the first part, studying the temporal and spatial variation of bacterial communities on the rhizosphere of chrysanthemum. They found that the predominant groups were related to bacteria from environmental samples (*Bacillus* spp.) and perhaps with bacterial genera associated with the rhizosphere (i.e., *Pseudomonas*, C*omamomas*, A*cetobacter* among others) (Duineveld et al., 1998). Another finding of this study was that dominant bacterial groups did not have the highest level of metabolic activity, an aspect that could be species specific. Young plants were found to contain bacterial communities distinct from plants at other developmental stages, implying microbial succession during plant growth (Duineveld et al., 2001). Still, it remains unknown how dominant bacterial groups change after steaming or how specific communities that are metabolically active could be affected over time.

Recently, the dynamics of key soil non-pathogenic microbial communities, especially those involved in nitrogen cycling, following steam disinfestation were studied. Roux-Michollet et al. (2008) showed that steaming induced significant decreases in community activity and size, and changes in community composition, with nitrifying bacteria being most affected (Roux-Michollet et al., 2008). This work was conducted over a 60 day period and no crop was cultivated or agricultural practice applied following steam disinfestations. Therefore, it is not possible to extrapolate these results for the potential effects of steaming on soil microbes in the

presence of plant roots. Short-term effects of steaming have also been examined for arthropods, although in these studies the steam treatment was done once and without cultivation of crops. Steam disinfestation has been widely used without having a good knowledge of long-term implications on soil communities in rhizosphere soil, such as how much can soil biota withstand disturbances, and whether or not community structure is affected. Effects of steaming can have important long-term consequences for the soil microbial communities, especially for bacteria and fungi. Hence, there is a critical need to investigate those effects.

Molecular techniques for analysis of soil microbial community structure

Among fingerprint techniques, terminal-restriction fragment length polymorphism (T-RFLP) analysis, automated ribosomal intergenic spacer analysis (ARISA), and denaturing gradient gel electrophoresis (DGGE) have been widely used for analysis of microbial communities (Okubo and Sugiyama, 2009). T-RFLP analysis is based on restriction endonuclease digestion of fluorescent end-labeled PCR products. The digested fragments are separated by electrophoresis and laser detection of the labeled fragments is done using an automated analyzer. T-RFLP have been used to study soil microbial structure and dynamics (Osborn et al, 2000), soil bacterial diversity (Smalla et al, 2007), spatial distribution of fungal communities (Robinson et al, 2009) and the effects of vegetation cover on soil community structure (Chim Chan et al, 2008).

ARISA exploits the variability in the length of the intergenic spacer (IGS) between the small (16S) and large (23S) subunit rDNA genes in the *rrn* operon. Like RFLP, ARISA involves the use of a fluorescence-tagged primer for PCR amplification and for subsequent electrophoresis in an automated system (Ranjard et al, 2001). This approach has been used to assess the structure of soil bacterial communities (Ranjard et al, 2000a) and to evaluate the

changes that follow antibiotic treatment (Robleto et al, 1998), mercury stress (Ranjard et al, 2000b), and deforestation (Borneman and Triplett, 1997).

In DGGE fingerprint technique, DNA fragments of the same length, but with different sequences, can be separated. Separation is based on the decreased electrophoretic mobility of a partially melted double stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide). The melting of DNA fragments proceeds in discrete so called *melting domains:* stretches of base pairs with an identical melting temperature. Once a domain with the lowest melting temperature reaches its melting temperature (*Tm*) at a particular position in the denaturing, transition of a helical to a partially melted molecule occurs, and migration of the molecule will practically halt. Sequence variation within such domains causes the melting temperatures to differ, and molecules with different sequences will stop migrating at different positions in the gel (Muyzer et al, 1997). DGGE has been used to analyze bacterial communities in different soil types (Suzuki et al, 2009), to study the effects of soil disinfestations on soil nitrogen dynamics (Roux-Michollet et al, 2008; Yamamoto et al, 2008), the effects of long-term application of fertilizer on soil microbial communities (Wenhui et al, 2007), to assess the diversity of rhizosphere communities of transgenic crops (Milling et al, 2004), and to analyze wood-inhabiting fungi and soil fungal communities (Vainio and Hantula, 2000; van Elsas et al, 2000).

In a comparison among T-RFLP, ARISA, and DGGE, Okubo and Sugiyama found that all of the methods are suitable to analyze soil communities. ARISA had the largest number of total bands for both fungal (Okubo and Sugiyama, 2009) and bacterial communities (Jones and Ties, 2007). ARISA had the highest resolution ability which makes it suitable for diversity analysis. T-RFLP produced intermediate discrimination between ARISA and DGGE; therefore it

can be used for analysis of communities consisting of specific taxonomic groups with low diversity. The fungal community profile obtained with DGGE had a lower number of bands, compared to T-RFLP and ARISA. However, DGGE analysis allows the extraction of DNA of a target band from the gel and sequence analysis through production of clone libraries, which is an advantage of this method over the other two methods for analyzing microbial communities (Okubo and Sugiyama, 2009).

Questions to be addressed

What bacterial groups are re-colonizing the rhizosphere first after steaming of soils? Which bacterial populations are predominant in the rhizosphere following steaming, and how different are these from populations in non-treated soil? Are there any effects of repetitive steam application on soil microbial communities? Are those effects reversible? Can those changes be remediated by applying *Bacillus* strains?

Are the aerobic endospore-forming bacteria the first re-colonizers if the plant is inoculated with them? Is the microbial diversity affected by this steam application? Are re-colonizers virulent? Are endophytes also affected by steam-treatment?

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Chapter II. Changes in the abundance of bacterial microbiota in the rhizosphere and roots of chrysanthemum after soil steaming

Abstract

Soil steaming is a substitute for fumigation with methyl bromide for managing soil-borne pathogens. Recently, growers of cut-flowers have noticed a reduction in flower size and weight, which they attribute to steaming the soil. Since shorter and lighter plants lose their commercial value and, therefore, are not accepted in the international market, there is a need to determine the effects of steaming soil on plants and their microbial communities. The specific purpose of this study was to evaluate the impact of soil steaming on soil microbial community abundance in the rhizosphere using culture-dependent methods and chrysanthemum as a model. To examine the effects of steaming on the rhizosphere microbiota, field and greenhouse experiments were conducted to monitor the changes after steam application of total culturable bacteria, fluorescent pseudomonads, and aerobic spore-forming bacteria (AEFB). For analysis of rhizosphere bacterial populations obtained in the field experiment, five treatments were evaluated for each of the following situations: 1. Non-treated field soil (soil that has never been steamed or treated with fumigants); 2. Soil steamed prior to planting; 3. One planting cycle following a single steam application; 4. A second planting cycle following a single steam application; and 5. A third planting cycle following a single steam application. Two planting cycles, each repeated once, were conducted in the greenhouse. In the first cycle of the greenhouse pot assay, treatments included steamed soil, steamed soil with PGPR amendment, and non-steamed soil. For the next cycle, half of the steamed soil was re-steamed, adding a fourth treatment. In the field evaluations,

there were significant increases of fluorescent pseudomonads in the rhizosphere with each planting cycle, even though steam was only applied prior to the first planting cycle. It is noteworthy that increases in populations of fluorescent pseudomonads corresponded to reduced growth of chrysanthemum. Results of the greenhouse assays showed that, in the first planting cycle, bacterial populations from non-steamed soil were significantly lower than populations from steamed soil and steamed soil with PGPR, a result that was unexpected. In the second planting cycle, steamed soil with PGPR had higher populations of total bacteria and AEFB than non-steamed soil. Soil that was steamed twice had significantly higher populations of fluorescent pseudomonads than all other treatments but had significantly lower populations of total bacteria than all other treatments. Populations in soil that was not re-steamed for the second cycle differed from the control, suggesting that populations will not return to control levels in that period of time even if steam is not reapplied. Our results show that repeated steaming of soil changes the abundance of the microbial community and that these changes are consistently associated with the reductions in plant growth observed by growers, suggesting a causal relationship.

Introduction

As chemical pesticides experience reductions in viability and usage due to genetic resistance of pest species (Orzech and Nichter, 2008), ineligibility due to "aggregate risk" (http://www.epa.gov/pesticides/trac/science/aggregate.pdf), and demands of consumers for organic products (Batte et al., 2007), the development of alternatives to traditional pesticides for management of soil pathogens and pests continues to increase in importance (Eshel et al., 2000; Gamliel et al., 2000). The phasing out of methyl bromide is a prime example of an effective chemical being banned due to its adverse effects on the environment and pressure from consumer

and advocacy groups for more environmentally-sound growing practices. Soil steaming is one alternative to chemical fumigants (such as methyl bromide) that has been adopted by growers, primarily because it achieves the goals of chemical fumigants without leaving toxic residues in the soil. Another benefit of steaming versus chemical application is that with steaming, planting may commence immediately after the soil has cooled down, without the delays necessary with chemical fumigants (Pizano, 2001).

Many of the community-level effects of soil steaming have been documented, including effects on weeds (Barberi et al., 2009; Melander and Jorgensen, 2005), soil recolonization by arthropods (Fenoglio et al., 2006), abundance of heterotrophic bacteria and soil community structure (Roux-Michollet et al., 2008), soil fungi (Awuah and Lorbeer, 1991), and soil properties (Egli et al., 2006). However, there is no consensus among researchers of the impact of this practice on the soil microbial community. Since temperature and moisture are widely known to affect growth and survival of bacteria (Braker et al., 2010; Szukics et al., 2010), it stands to reason that repeatedly applying steam to soil will affect the composition of the microbial community by selecting against some heat intolerant taxa, while allowing heat-tolerant taxa to proliferate. While some studies have shown potential effects of steaming on soil microbiota, all of these studies were done in soil steamed only once, some in which no crop was cultivated and no agricultural practice applied. Although growers typically use repetitive steaming, there are no reports on the effects of this practice on indigenous microbial communities in the rhizosphere or their recolonization process. Because there is a close and agriculturally essential relationship between plants and the soil micro flora it is vitally important to understand how the microbial community responds to soil steaming.

Soil steaming has been extensively used in the commercial floriculture industry to reduce populations of soil-borne plant pathogens (Pizano, 2001). For instance in Colombia, the country that exports the largest quantity of chrysanthemum to the USA, steam is applied to soil before preparation of the planting beds. In this system, the frequency of steam application has been increasing for two reasons. First, the crop is intended for export, where high quality standards demand disease-free cut flowers with a long shelf-life. Second, growers that previously used steaming have noted a reduction in height and weight of chrysanthemum plants when steam is not applied after each planting cycle (Juan Diego Aristizabal, personal communication). Since shorter and lighter plants lose their commercial value in the flower industry, they are not accepted in the international market. In addition to the field evaluations for this study, a greenhouse assay was conducted to evaluate under controlled conditions the effects of consecutive steaming on bacterial populations in the rhizosphere of chrysanthemum.

In other agricultural systems, high density production and monoculture have also been associated with changes in the structure of bacterial populations in soil, resulting in the dominance of certain deleterious or allelopathic groups that have negative effects for plant growth (Alström, 1987; Alström, 1992; Li and Kremer, 2000; Nehl et al., 1997; Schippers et al., 1987). In general, members of the bacterial genus *Pseudomonas* are the microorganisms most commonly reported as deleterious rhizobacteria (DRB) due to their association with plant growth reduction (Alström, 1987; Boel et al., 1993; Suslow and Schroth, 1982). DRB, also called allelopathic bacteria (Kremer, 2007), have been isolated from a variety of crops such as potatoes (Sturz *et al.*, 2000), carrots (Surette *et al.*, 2003), citrus (Gardner *et al.*, 1985), peas (Berggren *et al.*, 2001), and peach (Benizri *et al.*, 2005)) that exhibited abnormally reduced growth which

could not be associated with pathogens or nutritional deficiencies. An association among DRB and growth reduction in the Colombian Chrysanthemum has not yet been reported.

The overall aim of this study was to test the hypothesis that reduced growth of chrysanthemum, observed with repeated steaming of planting beds, is associated with increased populations of fluorescent pseudomonads. The specific objectives of the study were: 1) to quantify the effects of consecutive soil steaming on the bacterial populations in the rhizosphere of chrysanthemum under field and greenhouse conditions; 2) to establish whether the effects are cultivar dependent; 3) to determine the effect of steam-treatment on endophytes; and 4) to evaluate if PGPR application will promote chrysanthemum growth when the soil has been steamed.

Experimental approach

Studies to determine the effects of steam application on the abundance of bacterial populations in the rhizosphere soil were conducted under natural field conditions in a Colombian flower company (Miramonte y Flores la Esmeralda) and were also recreated under controlled greenhouse conditions in Alabama.

General procedures

To determine abundance of bacterial populations in the rhizosphere of chrysanthemum, populations of total aerobic culturable bacteria, fluorescent psuedomonads, and aerobic endospore forming bacteria (AEFB) were recorded by direct plate count. All populations were isolated from the rhizosphere of the cultivar White Vero, which was planted under agronomic management for commercial production in northwest Colombia and also from the cultivar Golden Kent, cultivated under greenhouse conditions in Alabama. White Vero could not be used in green house studies because it was not available from U.S.-based companies at the time of the

experiment. Soil microbial populations were surveyed 5 weeks after transplanting in the commercial setting and 5 and 10 weeks after transplanting in our greenhouse model system. At each sampling time the population counts were determined using the procedure described by (Burkett-Cadena et al., 2008). For the soil from Alabama, 10^{-1}, 10^{-2}, and 10^{-3} dilutions were plated for total populations and 10^{0} and 10^{-1} dilutions were plated to quantify pseudomonads and AEFB; for the Colombian soil the dilutions plated were 10^{-4} and 10^{-6} for total populations,10^{-3} and 10^{-4} for pseudomonads and AEFB. Numbers of colonies were counted, and population size was expressed as colony forming units (cfu)/g of dry soil for all treatments.

1. Field assays [1]

The field studies were performed using rhizosphere soil from planting beds of two commercial flower companies located in Antioquia, Colombia. In one of these companies, soil steaming is a frequently used practice to manage soil-borne pathogens, and reduction in growth and weight of chrysanthemum has been observed when steam in not applied regularly. On this farm, soil steaming is usually done using a polyethylene sheet to cover the planting beds that are sealed at the edges. The steam produced by a boiler is blown through a pipe under the plastic sheet for one hour. The soil temperature reaches 85°C at the surface. After steaming, the soil is aerated for 24 hours before planting. Because in this flower company there was not a single planting bed without a history of steaming, we used soil from another flower company located in the vicinity as a non-steamed control. This non-steamed farm did not use any other physical or chemical method for soil disinfection nor reported any reduction in chrysanthemum growth. In both companies, the same soil type (Andisol) was present and fertilization was conducted according to soil analysis and the nutrient requirements of chrysanthemum.

[1] Field data was collected and analyzed by Camilo Ramirez and collaborators from Universidad de Antioquia in 2008.

For analysis of rhizosphere bacterial populations, five treatments with 15 replicates were evaluated: 1) untreated field soil (soil that has never been steam-treated); 2) soil steaming prior to planting; 3) one planting cycle after the soil was steamed only once; 4) a second planting cycle after the soil was steamed once; and 5) a third planting cycle after the soil was steamed once. Each planting cycle consisted of a 12-week period from transplanting the chrysanthemum seedlings into the planting bed until harvesting.

Sampling

Samples of rhizosphere soil were taken 5 weeks after transplanting. The samples were obtained from the rhizosphere of the chrysanthemum cultivar White Vero. Twenty plants of chrysanthemum were removed from each bed following a zigzag pattern, and the soil adhering to the roots was analyzed as rhizosphere soil. Plants from the edges of the bed were not included. Rhizosphere soil from a total of fifteen beds per treatment was collected, placed in a Ziploc plastic bag, sealed and shipped to University of Antioquia for analysis. Chrysanthemum plant growth parameters, height and weight, were recorded for each of the plants sampled.

Statistical analysis

All analyses were conducted with the GLIMMIX procedure of SAS® (SAS Institute, NC), following a completely randomized (CRD) design and, therefore, with the pooled residual within each treatment as the residual term. First, normality and homogeneity of variance assumptions were tested using the student panel graphs generated by proc GLIMMIX. Normality was only warranted for plant weight, while plant height and total bacteria, pseudomonads, and AEFB populations had a log-normal distribution. Homoscedasticity was only fulfilled for plant weight and the $\log_e$ transformation of total culturable bacteria. For the rest of response variables ($\log_e$ transformed), the variance structures were modeled (*R*-side of the covariance parameter of

SAS) using the group option to create homogeneous variance groups for each treatment. A smaller AIC value from the 'information criteria' output and a better graphical residual distribution were considered as indicators of a good fit for the model. Comparison of means was conducted by using the simulate adjustment of proc GLIMMIX and is presented in the graphs as significant classes.

2. Greenhouse assays

Steam treatment

A novel device was designed and constructed for steaming soil in quantities of 20 Kg. The device consisted of a metal cylinder, 1.60 m high and 0.15 m diameter, attached at its base to a steam generator via a copper pipe. Steam from the generator was blown through the pipe so as to penetrate the soil from the bottom of the cylinder. Soil was steamed in this manner for 40 min at >80 °C. A small hole, drilled midway along the length of the cylinder allowed a thermometer to be inserted into the soil to monitor core temperatures.

Soil and plant material

Field soil, collected from E. V. Smith Agricultural Research Station in Alabama, was mixed with Sunshine mix ®(Sun Gro Horticulture, Bellevue, WA) 1:1 v/v, which I will refer to as soil from this point. Soil samples were sent to the Auburn University Soil Testing Laboratory for soil analysis. Based on nutrient contents of the soil, an appropriate fertilization program was planned and pH adjusted to 6.5. One-month-old chrysanthemum seedlings cultivar golden Kent were used for all trials and four seedlings were planted in each 1-liter pot previously filled with steam-treated soil or with non-steamed soil. Plants were grown at 23°C and a photoperiod of 16 h light and 8 h of darkness. The plants were watered daily, soil moisture was maintained at 60% of its water holding capacity, and plants were fertilized with 20-10-20 at 4.05grams per pot twice

per week. The fifth week after transplanting the daylight regime was changed from 16 h to 12 h light to induce flowering. Growth cycles were terminated 10 weeks after planting.

Sampling

Samples of rhizosphere soil from two chrysanthemum plants were taken at 5 and 10 weeks after transplanting. Plants were removed from pots and the soil adhering to the roots was considered as the rhizosphere soil sample. Soil from each group of plants was placed in a Ziploc plastic bag and subjected to bacterial population analysis. For the second planting cycle, half the steamed soil was re-steamed, adding a fourth treatment. After replanting the soil with new seedlings for the second cycle the sampling was done at the same time interval.

Treatments

The experiment was conducted utilizing a completely randomized design (CRD) with two consecutive planting cycles. Three treatments with 6 replicates each were applied as follows for the first planting cycle: 1) control, non-steamed soil; 2) soil steamed at first cropping cycle; 3) Soil steamed at first cropping cycle plus the addition of a mixture PGPR. For the second planting cycle the treatments were: 1) control, second planting non-steamed soil; 2) second planting of soil steamed once; 3) second planting of soil steamed once plus the addition of the same mixture of PGPR as in the previous cycle; and 4) soil steamed twice. Chrysanthemum plant weight was recorded for each plant sampled. For the PGPR treatment, chrysanthemum plants were dipped in a mixture of four *Bacillus* strains, *Bacillus pumilus* INR7, *B. amyloliquefasciens* FZB42, *B. amyloliquefasciens* MBI600, *B. amyloliquefasciens* GB03, each applied at a concentration of 1x 10^7 cfu/ml. PGPR strains were selected because individually they were highly antagonistic against pseudomonads isolated from a different environment and

because they are commonly used as the active ingredient in commercial soil inoculants. Consequently, we wanted to evaluate their performance in soil conditions.

Isolation of endophytes

Chrysanthemum roots were surface sterilized by dipping them in 80% ethanol for 5 min, washing them in sterile, distilled water three times, bleach-sterilizing in 3% sodium hypochlorite for 30 s, and finally washing them in sterile distilled water ten times. Effective removal of epiphytes was tested by incubation of 50 µL of the washing water onto TSA for 48 hours at 26°C. Lack of bacterial growth on TSA plates inoculated with washing water verified that the outer surface sterilization effectively eliminated the outer surface bacteria. For the isolation of endophytic bacteria, total culturable bacteria, fluorescent pseudomonads, and AEFB, roots tissue extracts were serially diluted in sterile distilled water and plated on 50 % TSA or 50% King's B. Plates were incubated for 48 hours at 28 °C. Populations were counted and expressed as CFU per gram of fresh root weight.

16S rRNA gene sequencing for identification of fluorescent pseudomonads

A number of colonies of Pseudomonads from the rhizosphere and endophytes were randomly selected per treatment for identification based on ribosomal DNA. A single bacterial colony was picked from the 50% King's B agar plate, boiled in 20 µl distilled water for 7 min, and this solution was immediately used in PCR. The primer set used was 8F and 1401R. The reaction mixture consisted of 6 µL of template and 44 µL of PCR mix. The PCR mix was as follows: 25 µl of EconoTaq® plus green 2X Master Mix (Lucigen Corporation, Middleton, WI, USA), 0.5 µL of each primer (20 µM, Invitrogen Corporation, CA, USA) and 19 µL PCR certified water (Teknova, CA, USA). An initial denaturation of 5 min at 95 °C was followed by 31 cycles of denaturation of 1 min at 94 °C, annealing of 45 sec at 57 °C, and primer extension

of 2 min at 70 °C. PCR was finished with a final extension of 8 min at 70 °C. PCR products were checked by electrophoresis in 1.0 % (w/v) agarose gel, followed by ethidium bromide staining. PCR products were sent for sequencing to the Lucigen Corporation (Middleton, WI), and nearest neighbor identification for the fluorescent pseudomonads was obtained with the basic local alignment search tool (BLASTN) (Altschul et al., 1990).

Statistical analysis

Response data from the greenhouse experiment were analyzed as a factorial with cycle and treatment as factors; effects for experiment and repetition within experiment were subsumed in the residual error term. The distributional assumption was first evaluated using the student panel graphs available in SAS® Proc GLIMMIX. The normality assumption was acceptable only for plant weight. All other response variables followed a log normal distribution. The variance structure of the residual term was modeled using the group option to create homogeneous variance groups. Contrasts were used to assess the differences among treatments. Actual adjusted *P*-values are presented in the tables.

Results

1. Field assays

Effect of steaming on abundance of culturable bacterial populations in the rhizosphere of chrysanthemum

Results of field evaluations indicated that steaming the soil produced significant changes in populations of culturable bacteria. Immediately after soil was steamed, and before transplanting of chrysanthemum into the soil, total bacteria, and fluorescent pseudomonads populations were reduced; however, the most dramatic reduction occurred in pseudomonads (comparison of the recently steamed soil and the same soil after several planting cycles in Figure

1). Steaming never resulted in total elimination of culturable bacteria or fluorescent pseudomonads immediately following steam application (Figure 1).

Once the soil was planted with chrysanthemum, total bacteria populations in the rhizosphere were similar across all three planting cycles after steaming, but were significantly greater than in recently steamed and non-steamed (control) soil (Figure 1). Interestingly, for the first planting cycle, populations of fluorescent pseudomonads were similar in the soil where steam was applied and where steam was never used (Figure 1). However, populations of this group were greater in steamed soil after the first planting and continued to increase in the rhizosphere of chrysanthemum with each successive cycle, *i.e.*, they were significantly higher in the second and third planting cycles compared to the first cycle and to the soil where steam was never applied (Figure 1). Populations of AEFB were significantly greater in most of the treatments with steamed soil than in non-steamed soil. However, in contrast to fluorescent pseudomonads, no significant increase in the AEFB populations was found with consecutive planting cycles.

Along with the changes in bacterial populations found in the field, chrysanthemum height and weight showed differences after several planting cycles following soil steam treatment. Significantly taller and heavier plants resulted from planting in non-steamed soil, compared to plants grown in steamed soil. In addition, further reductions in height and weight were observed in the second and third planting cycle after steam application (Figure 2).

2. Greenhouse Assays

Total heterotrophic bacterial counts (which I will refer to hereafter as total population), AEFB and fluorescent pseudomonads were initially evaluated in the non-steamed and steamed soil immediately after steaming (Figure 4). Total bacterial counts and fluorescent pseudomonads

were significantly decreased in the steamed soil compared to the control. Similar reduction but not as drastic was observed for AEFB. A second evaluation of the populations was done in the soil of all the treatments including the soil re-steamed before planting the second cycle. As with the previous steam application all the populations were reduced in the treatment where steam was reapplied. Total bacterial populations and pseudomonads remained higher in the treatments with a single steam application, while AEFB were only higher in the steamed with PGPR treatment (Figure 5).

Effect of steaming on the abundance of culturable bacteria in the rhizosphere of chrysanthemum

In greenhouse pot assays, total populations, AEFB, and fluorescent pseudomonads were recorded five and ten weeks after transplanting for two consecutive planting cycles (cycle 1 and cycle 2). In cycle 1, total populations in the rhizosphere of chrysanthemum were greater in steamed soil and in soil steamed plus the addition of PGPR, compared to the non-steamed control (Table 1). Populations of total bacteria were not significantly different from steamed soil and steamed soil with PGPR at five weeks after transplanting; indicating that adding PGPR to steamed soil does not result in an overall increase in total culturable bacteria. In the second planting cycle, populations of total bacteria from the rhizosphere of chrysanthemum grown in non-steamed soil and soil steamed twice were not significantly different. However, both populations from these treatments were lower compared to those from rhizosphere of plants grown in soil steamed once and in soil steamed once + PGPR (Table 1). None of the treatments differed significantly between cycles, with respect to total population.

Populations of fluorescent pseudomonads were significantly greater in treatments with steamed soil and steamed soil + PGPR, compared to the control in the first planting cycle (Table

1). There was no significant difference between pseudomonad populations in soil steamed once and soil steamed once plus PGPR. From the first to the second planting cycle, populations of pseudomonads decreased to undetectable levels in all treatments where the soil was steamed once and also in the non-steamed control. In cycle 2, pseudomonads were only found in soil steamed twice.

For the first planting cycle, the population of AEFB in rhizosphere soil of the control was not significantly different from that of steam + PGPR (Table 1). Soil steamed once had significantly lower populations of AEFB than the other two treatments, suggesting that steaming reduced populations of spore-forming bacteria in the rhizosphere. In the second planting cycle populations were significantly higher for the treatment with PGPR than all other treatments. Populations of AEFB were significantly higher in soil steamed once than in soil steamed twice. Plant fresh shoot weight increased for treatments where steam was applied compared to the untreated control in cycle 1 (Figure 6). However, there were no significant differences in weight among treatments for the second planting cycle.

Effect of steaming on abundance of endophytic culturable bacteria in chrysanthemum

In the first planting cycle, total populations of endophytes were highest in soil steamed once + PGPR; however, this population was not significantly different from the population for the control (Table 2). For cycle 2 populations of endophytes from treatments with soil steamed once were significantly higher than those with soil steamed twice. Populations from treatments with soil steamed twice were also higher compared to the control (Table 2).

The effect of steam on populations of endophytic psseudomonads differed from those of total endophytic bacteria. In the first planting cycle, populations of fluorescent pseudomonads

were significantly lower for the treatment with PGPR than for the steamed treatment (Table 2). For the second planting cycle pseudomonads were not recovered as endophytes.

In cycle 1, populations of AEFB were significantly higher in the treatment with steamed soil + PGPR than steamed soil and non-steamed soil. During the second planting cycle populations of AEFB in the treatment with steamed soil + PGPR were significantly higher than those of all other treatments. Additionally, steaming the soil twice resulted in the reduction of endophytic AEFB, as in the rhizosphere (Table 2).

16S rRNA gene sequencing for identification of fluorescent pseudomonads

A number of pseudomonads were isolated from the rhizosphere and from inside the roots of chrysanthemum (Table 3, Figure 7). Some bacterial species were adversely affected by steam treatment and were never recovered from steam-treated soils, while other species proliferated in response to steam application.

For cycle 1 at five weeks sampling, 70% of the species in the non-steamed control corresponded to *P. putida*, 25% to *P. fluorescens*, and 5 % to *P. monteilii*. The treatment with steamed soil had an increase in the proportion of *P. fluorescens* and a decrease in *P. putida* compared to the control. Additionally, *P. fulva*, and *P. monteilii* were found, but in low numbers. For the treatment of soil steamed once + PGPR, *P. putida, P. fluorescens*, and *P. jessenlii* were predominant (Table 3).

At the ten week sampling for the first planting cycle, the relative abundance of bacterial species changed with respect to the week 5 sampling in the rhizosphere soil. In the control, *P. putida* decreased from one sampling to the next, while *P. mosselii* increased in proportion. The contrary effect was found for the steamed treatment, as *P. putida* increased and *P. fluorescens*

was not found. For the treatment with PGPR, *P. putida* and *P. citronellolis* increased in the week ten sampling.

For the second planting cycle, pseudomonads were found in the treatment with soil steamed twice, but not in any other treatment. Fourteen isolations were made, all of which were identified as *P. citronellolis*.

For the entophytes, *P. citronellolis*, *P. fluorescens*, and *P. putida* were the species found among treatments at five and ten weeks sampling of the first planting cycle (Figure5). In steamed treatment *P. putida* decreased in proportion compared to the control, while *P. fluorescens* increased in proportion. For the treatment with PGPR, *P. putida* and *P. citronellolis* increased compared to the control. In the second cropping cycle endophytic fluorescent pseudomonads were below detection limit.

Discussion

The overall aim of this study was to test the hypothesis that reduced growth of chrysanthemum, observed with repeated steaming of planting beds, is associated with increased populations of fluorescent pseudomonads. Our results showed reductions in bacterial populations immediately following steam application, and quick recolonization, particularly by fluorescent pseudomonads after soil is planted with chrysanthemum. Although, the field and greenhouse studies demonstrated that steaming soil results in changes in the relative abundance and community structure of culturable rhizosphere and endophytic bacteria. Only in the field evaluations conducted in commercial flower companies in northwest Colombia growth reductions of chrysanthemum after the first planting cycle following soil treatment with steam were correlated (Figure 3) to changes in populations of fluorescent pseudomonads. Additionally,

for both the field and the greenhouse pot assays, growth effects in plants were not related to any pathogen in the soil, a result which was expected since steaming kills soil-borne pathogens.

In the field evaluations, we observed significant increases of fluorescent pseudomonads in the rhizosphere with consecutive planting cycles, even though steam was only applied prior to the first planting cycle (Figure 1). Noteworthy is that increases in pseudomonad populations were highly correlated to chrysanthemum growth reduction suggesting that the increases in populations of pseudomonads may result in deleterious consequences for plants. It has been reported that fluorescent pseudomonads can be deleterious in other agricultural systems (Kloepper et al., 2010; Kremer, 2007). In general, pseudomonads are a widely distributed group in agricultural soils in which survival and colonization of the roots increase rapidly (McSpadden, 2007; Raaijmakers and Weller, 2001). Another main group associated with plant roots is the bacilli, which are also well adapted to the rhizosphere (Weller, 2007).

Given that bacilli and fluorescent pseudomonads both colonize roots, we hypothesized that application of PGPR-bacillus strains with antagonism *in vitro* to fluorescent pseudomonads could lessen the increases in populations of pseudomonads associated with steaming. To test this hypothesis and to recreate under controlled conditions the system used in Colombia, a set of greenhouse pot assays was evaluated under control conditions in Alabama. Results similar to the field assays were obtained in the greenhouse pot assays. Specifically, that changes in rhizobacteria were due to steaming the soil. As in the field evaluations, increases in fluorescent pseudomonads were found in the soil treated with steam. However, growth reduction was not observed with repeated steam application.

For the first planting cycle in the greenhouse pot assays, populations of pseudomonads increased in response to steam application. During the second planting cycle these changes were

only recorded for the soil that was consecutively steamed, and these results differ from those found in the field, in which pseudomonads were also significantly higher in the second planting cycle after steam treatment. In addition, the soil used in the greenhouse study had lower indigenous populations of pseudomonads compared to the Colombian soil, suggesting that natural pseudomonad populations are influenced by soil type (reference). A number of studies have reported the effect of disturbance on the composition of soil microbial communities (Allison and Martiny, 2008). The application of steam is a type, albeit extreme, of temperature disturbance and thus the microbes of some agro ecosystems may have a faster recovery rate compared to others. Based on our results, and taking into account the abundance of one main group from the rhizospheric bacterial community, bacterial communities in the Alabamian agroecosystem can quickly return to their composition prior to disturbance in the second cycle after steaming. The opposite situation could be happening in the Colombian agroecosystem in which the relative abundance of the indigenous pseudomonads is higher due to greater organic matter content in the soil (120 %). Pseudomonads have a rapid growth rate; so if their abundance is lowered by a disturbance, they have the potential to recover quickly. In the case of the pseudomonads in the field, they not only recover, but increase their abundance to a level higher than their original in the community.

Additionally, increases in pseudomonads persist throughout several planting cycles and this fact may contribute to the chrysanthemum growth reduction and consequently to the high frequency of steam application needed in the flower industry. Apparently, populations of pseudomonads are reduced by steam prior to planting, but once the chrysanthemum plants are in the soil, the pseudomonads can quickly recolonize and increase their population to a level that is harmful to some chrysanthemum cultivars. Pseudomonads are known to have good root

colonizing capacities and have also received special attention because they are the most common taxon of microorganism found in the roots (Kluepfel, 1993) Probably, as found in other systems (Kloepper et al., 2010; Kremer, 2007), monoculture, continuous planting, and soil steaming may select for these populations in the rhizosphere of chrysanthemum.

Another major difference between the field and the greenhouse assays was that reduction in shoot weight was not observed for the later in the second planting cycle. The chrysanthemum plants grown in steamed soil and steamed soil +PGPR were heavier than the control only in the first planting cycle. Growth promotion as a result of soil steaming has also been recorded in other crops such as tomato, but only in the first planting cycle after steam application (Tanaka et al., 2003). The advantages of growing chrysanthemum in the tropics (Colombia) are related to environmental conditions. Temperature and photoperiod are critical to obtain taller and heavier plants. Although, in the greenhouse pot assay, light was modified to satisfy the number of hours demanded of chrysanthemum, those artificial conditions will never be equal to the ones found in the tropics. Therefore the lack of effects of consecutive soil steaming on chrysanthemum growth could be a consequence of the location more than the treatment itself.

This finding about the lack of effect on growth and the fact that pseudomonads did not increase in the soil steamed once for the second planting cycle may also be explained by a cultivar effect. The cultivar used for the greenhouse pot assay is not reported as susceptible by the Colombian growers, which means that growth reduction has not been observed in the field, contrary to the cultivar from which rhizosphere soil was obtained in the field evaluations. These results suggest that the microbial properties of the soil play a key role in the recovery process after disturbance. Additionally, there are some cultivars that will influence greatly the abundance

of pseudomonads in their rhizosphere, but only for some of them are the effects adverse for their growth. The cultivar effect needs to be studied in more detail.

The PGPR-bacillus mixture did not have an effect on populations of pseudomonads at any sampling time. Both groups were found equally in the rhizosphere of chrysanthemum, and application of PGPR did not reduce populations of pseudomonads. PGPR colonized the root system, as reported for other introduced bacteria (Compant et al, 2010), without changing the overall abundance of pseudomonads, but did have a significant effect on the relative abundance of *Pseudomonas* species (Table 4), reducing the percentage of *P. fluorescens* from 92% to 20%. PGPR mixture increased the AEFB in the rhizosphere of chrysanthemum while consecutive steaming decreased them.

The overall effect of soil steaming on the rhizosphere microbiota was to change populations of total bacteria and pseudomonads. Given that pseudomonads were the group most quickly to change in abundance, we wanted to evaluate if these changes in *Pseudomonas* were also accompanied by difference in species composition. The identification of the pseudomonads isolated from each treatment revealed that *P. fluorescens, P. putida*, and *P. monteilii* were the main species present in the first cycle of the greenhouse pot assays. In summary, for the pseudomonads in the rhizosphere, steaming the soil changed the proportion of the predominant groups as follows: increased the level of *P. putida*, caused fluctuations in the other pseudomonads species in the first cycle, and at the end of the second cycle 100% of the isolates corresponded to *P. citronellolis*. The identification of the fluorescent pseudomonads isolates showed that steaming not only changed the abundance of groups, but also the proportion and species composition in the rhizosphere of chrysanthemum.

Populations of endophytes were also recorded for the greenhouse pot assays. Results indicated that steaming does not change total population or pseudomonads within the roots. Pseudomonads were not found in the second cycle, indicating that the effect of steaming is established mainly in the rhizosphere. The effect of steaming on endophytes was primarily found in the treatment with PGPR during the first cycle. At five weeks sampling PGPR reduced endophytic pseudomonads, compared to the control, but at 10 weeks sampling PGPR had the opposite effect. This could be explained by the change in community composition of the pseudomonads. PGPR application changed the composition of pseudomonads by reducing the proportion of *P. fluorescen*sas in the rhizosphere. Additionally, PGPR increased the AEFB endophytes, suggesting that the PGPR were effective not only colonizing the root system, but also the inside of the roots, good evidence of endophytic colonization by PGPR-bacillus. Although pseudomonads found as endophytes in the first cycle also exhibited changes in species relative abundance in all treatments, there was a dominant endophytic species, *P. citronellolis,* at the end of the cycle. In addition, this was the only species found in the rhizosphere for the second planting cycle.

Hence, using PGPR, which exhibited antagonism against diverse fluorescent pseudomonads in *in vitro* studies, decreased initial endophytic populations of pseudomonads and caused shifts in their endophytic community structure. These findings lend support to the application of bacilli PGPR as inputs into management options for diseases such as fern distortion syndrome (Kloepper et al., 2010) that are associated with increased populations of fluorescent pseudomonads.

Analysis of the chemical properties of the steamed soil revealed that steaming does not change the soil pH (Appendix 7), suggesting that a mechanism other than pH drives the changes

in bacterial communities in steamed soil. Steaming changed mainly the levels of manganese, an effect previously reported (Sonneveld, 1979). However, the increased level of manganese was not toxic to the chrysanthemum plants or influential of the culturable bacterial community structure, indicating that changes in Mn levels do not drive changes in either plant characteristics or microbe populations.

There are many ways in which steaming the soil can affect the microbial properties of the soil. We found that steaming the soil increases the abundance of one of the main rhizosphere groups, the fluorescent pseudomonads, and that those changes are related to chrysanthemum growth reduction in the field. Additionally, PGPR did not vary the abundance of fluorescent pseudomonads, but did exhibit rhizosphere and endophytic colonization. How can this information modify the frequency of steam application? This can be accomplished by being aware that utilizing this so called environmentally friendly approach is also modifying the composition of microbes in the agroecosystem where it is delivered, and therefore that there is always a need for more research on the responses of microbial groups to disturbances. Finally there is an enormous variety of microorganisms that can be used to ameliorate the consequences.

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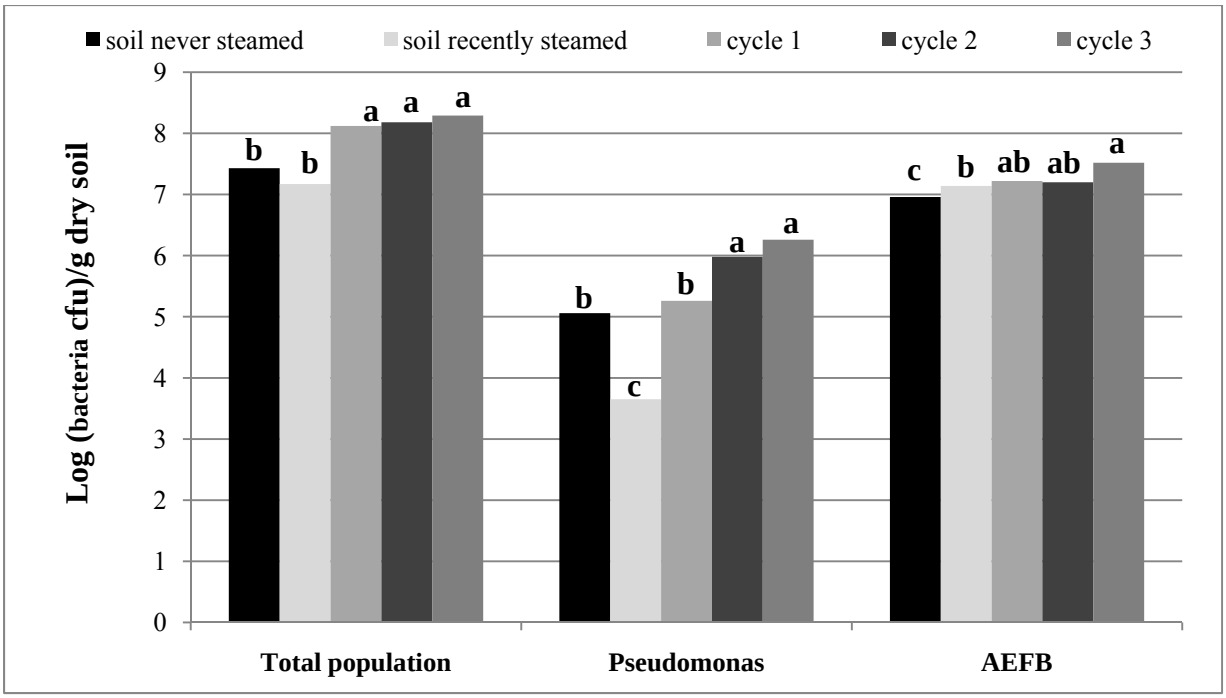


Figure 1. Field trial[1]. Plate count numbers (log transformation) at five weeks after transplanting for total culturable bacterial populations, fluorescent pseudomonads, and aerobic endospore forming bacteria (AEFB) present in rhizosphere soil. Treatments with the same letter within the same response variable are not significantly different (*P*-value > 0.05) according to simulate adjustment of GLIMMIX procedure. *F* tests had 66, 57, and 69 degrees of freedom for total bacteria, pseudomonads, and AEFB, respectively, and, in all cases, *P*-values were < 0.001. Trial was conducted under agronomic conditions for commercial production of chrysanthemum with cultivar White Vero. Cycle1 =steamed soil once first planting cycle. Cycle 2= steamed soil once second planting cycle, Cycle 3= steamed soil once third planting cycle.

[1] Field data was collected and analyzed by Camilo Ramirez and collaborators from Universidad de Antioquia.

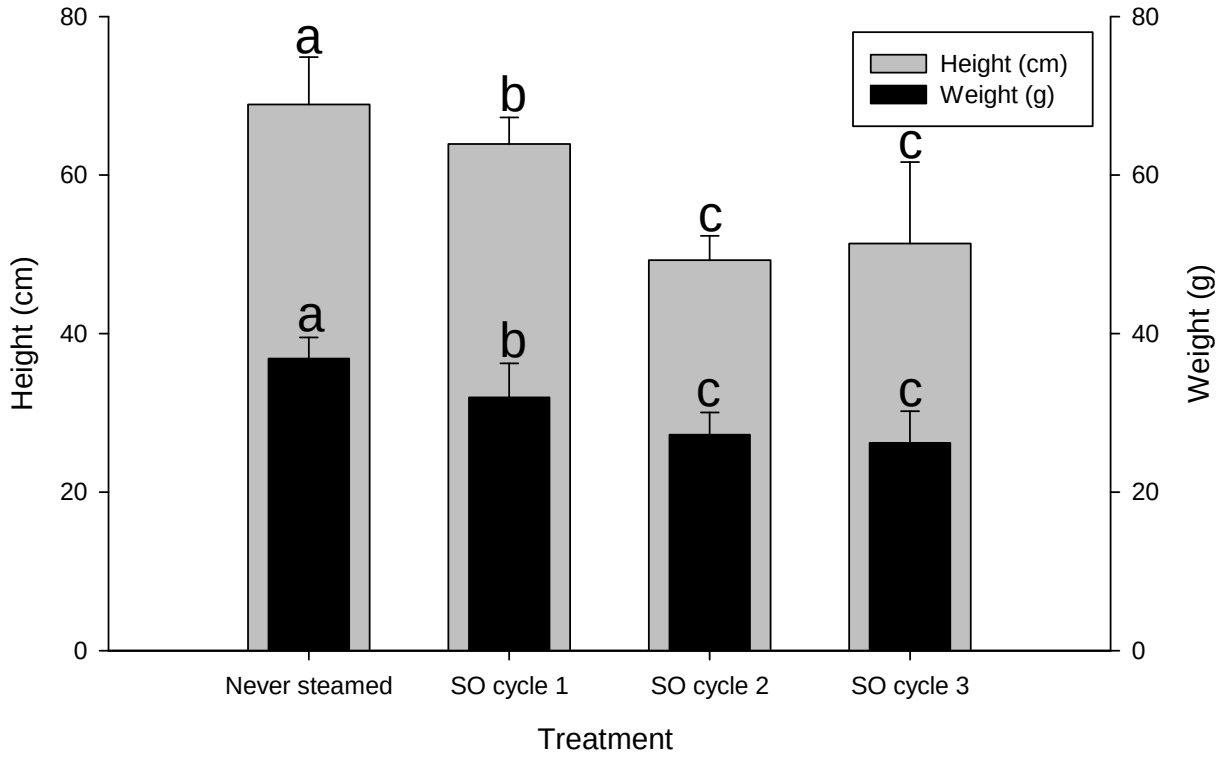
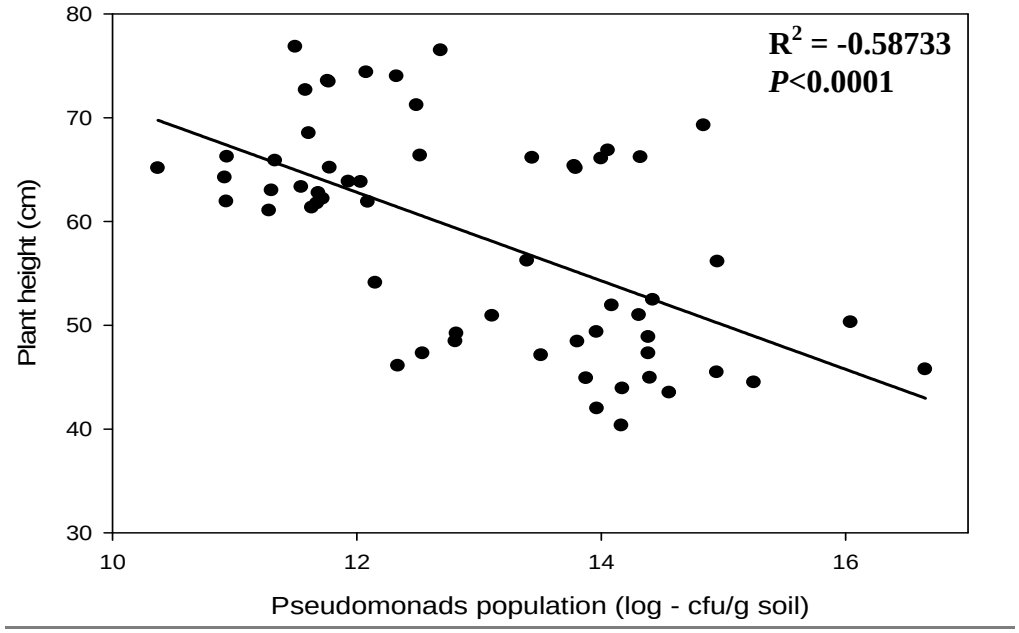


Figure 2. Field trial[1]. Height and weight of chrysanthemum plants at 5 weeks after transplanting. Means with the same letter within each response variable are not significantly different (*P*-value > 0.05) according to simulate adjustment of GLIMMIX procedure. Error bars represent the standard errors. *F* test had 56 degrees of freedom and a *P*-value < 0.001 for both response variables. Trial was conducted under agronomic conditions for commercial production of chrysanthemum with the cultivar White Vero. SO = soil steamed once.

[1] Field data was collected and analyzed by Camilo Ramirez and collaborators from Universidad de Antioquia.



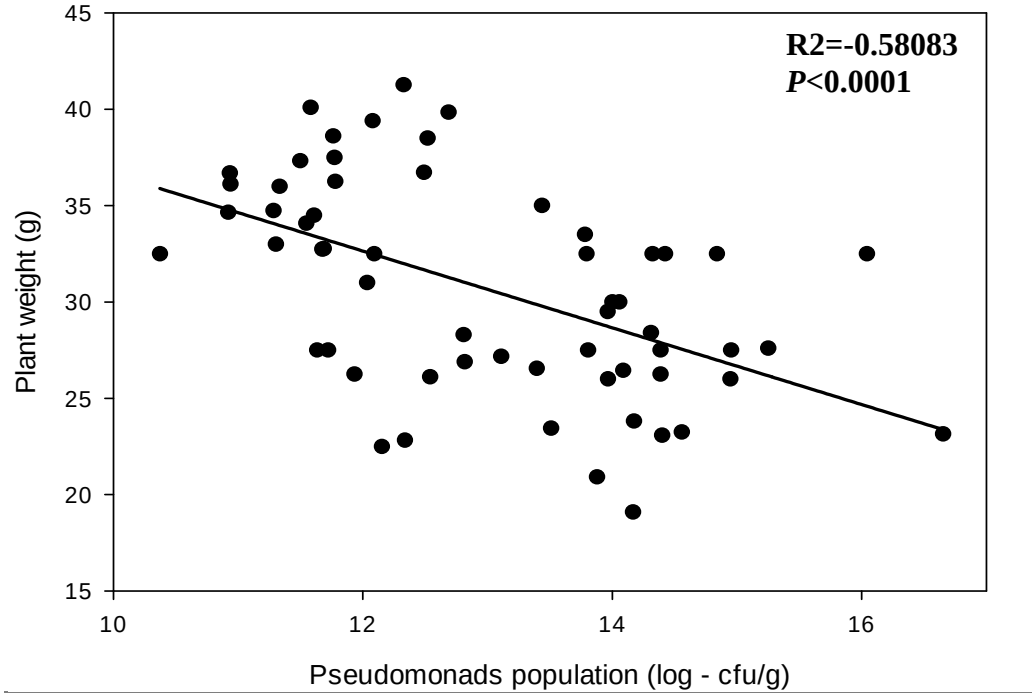


Figure 3. Field trial[1]. Correlation of chrysanthemum height and weight with pseudomonad populations in the rhizosphere soil.

[1] Field data was collected and analyzed by Camilo Ramirez and collaborators from Universidad de Antioquia.

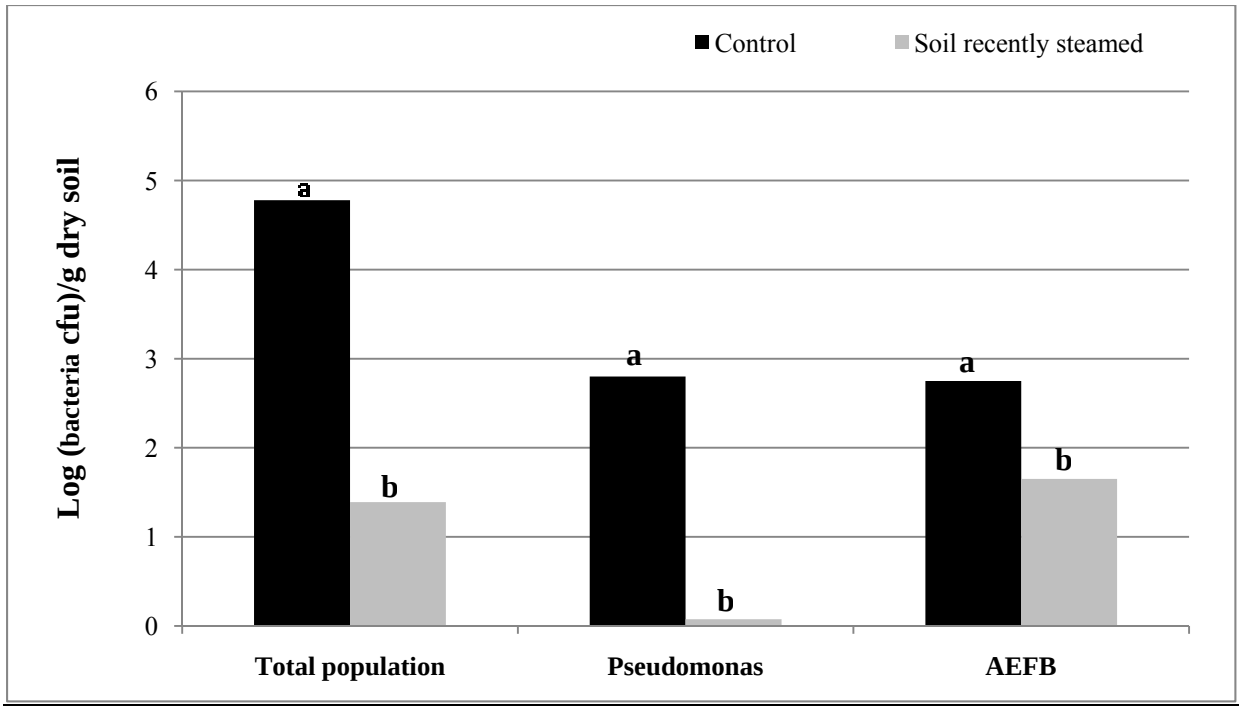


Figure 4. Greenhouse assay. Variation in plate count numbers of soil after steam application for total culturable bacterial populations, fluorescent pseudomonads, and aerobic endospore forming bacteria (AEFB). Treatments with different letters differ significantly at $\alpha=0.05$. Control = non-steamed soil before planting first cycle. Soil recently steamed= steamed soil before planting first cycle.

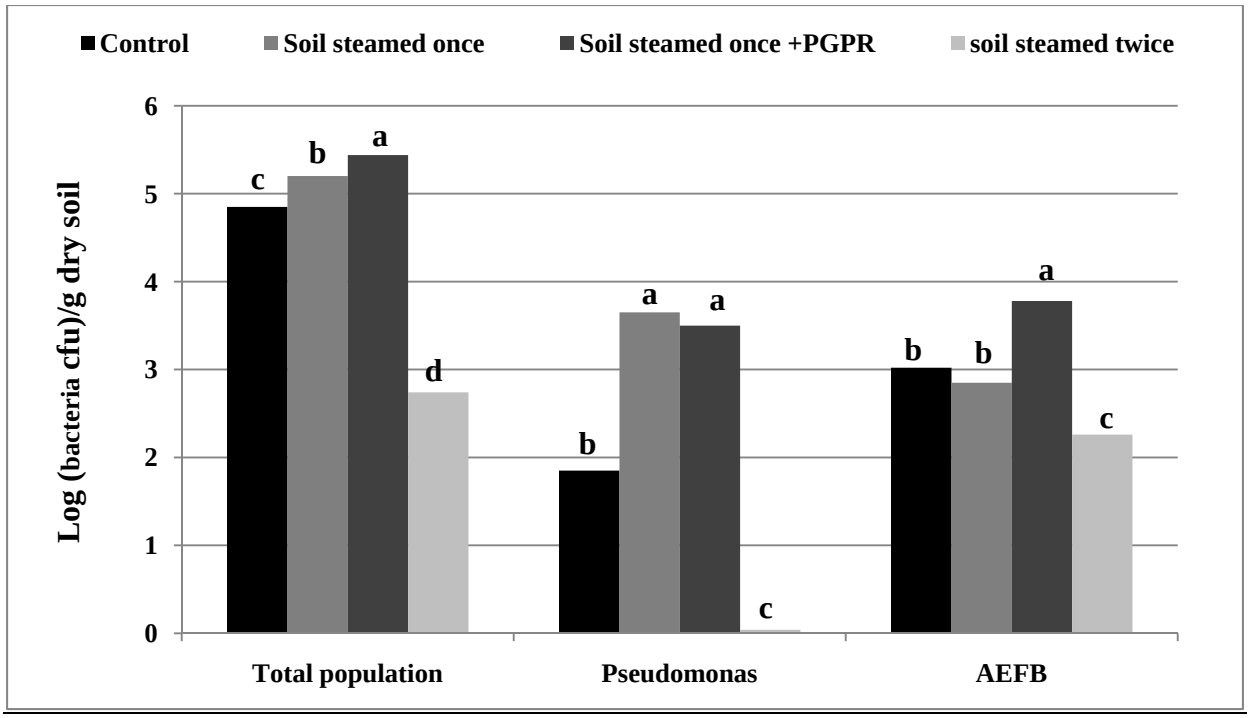


Figure 5. Greenhouse assay. Variation in plate count numbers of soil after steam application for total culturable bacterial populations, fluorescent pseudomonads, and aerobic endospore forming bacteria (AEFB). Treatments with different letters differ significantly at $\alpha=0.05$. Control = non-steamed soil after harvesting first cycle. Soil steamed once = steamed soil after harvesting first cycle. Soil steamed + PGPR = steamed soil after harvesting first cycle. Soil steamed twice =soil recently steamed after harvesting first cycle.

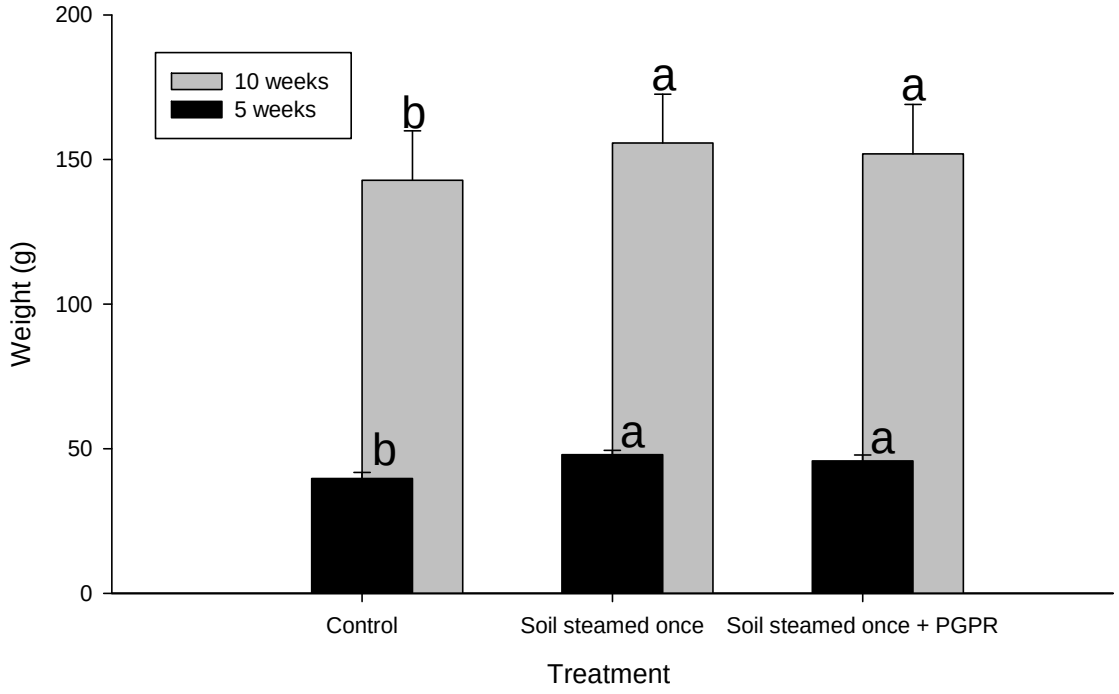


Figure 6. Greenhouse assay. Weight of chrysanthemum plants at 5 weeks and 10 weeks after transplanting for cycle 1. Means with the same letter within each response variable are not significantly different (*P*-value > 0.05) according to simulate adjustment of GLIMMIX procedure. Error bars represent the standard errors. Assay was conducted under controlled conditions with the cultivar Golden Kent.

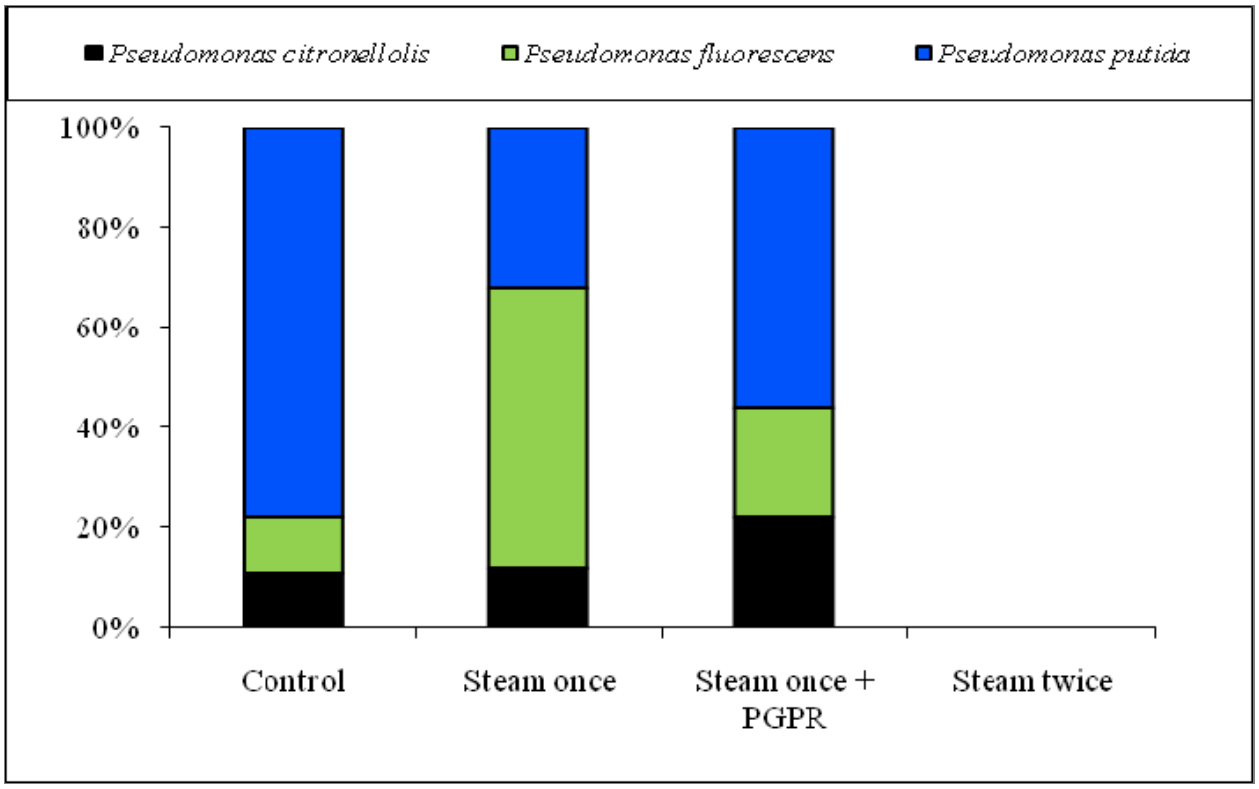


Figure 7. **Greenhouse assay**. BLAST nearest neighbor identification for the endophytic fluorescent *Pseudomonas* isolated from chrysanthemum using 16S rRNA sequencing. Percent of total isolates identified to species level for first cropping cycle.

Table 1. Greenhouse assay. Least square means and standard errors (SE) of bacterial populations at five weeks after transplanting for cycle 1 and ten weeks after transplanting for cycle 2, for total bacteria, fluorescent pseudomonads, and aerobic endospore forming bacteria (AEFB) of the rhizosphere of chrysanthemum. Treatments with different letters differ significantly at α=0.05.

	log(Total Bacteria cfu), g^{-1} dry soil				log(Pseudomonads cfu), g^{-1} dry soil				log(AEFB cfu), g^{-1} dry soil			
	Cycle 1		Cycle 2		Cycle 1		Cycle 2		Cycle 1		Cycle 2	
Treatment	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1. Control	6.68**b**	0.51	5.69**b**	0.13	4.26**b**	0.38	0	0	4.47**a**	0.13	3.84**b**	1.02
2. Soil steamed once	6.85**a**	0.51	6.39**a**	0.19	4.85**a**	0.37	0	0	4.24**b**	0.10	3.88**b**	1.02
3. Soil steamed once + PGPR	6.87**a**	0.51	6.26**b**	0.19	5.04**a**	0.38	0	0	4.47**a**	0.13	5.16**a**	1.02
4. Soil steamed twice			5.76**b**	0.19			5.1**a**	0.08			3.73**b**	1.02

Table 2. Greenhouse assay. Least square means and standard errors (SE) of bacterial populations at five weeks after transplanting for cycle 1 and ten weeks after transplanting for cycle 2, for total endophytic bacteria, endophytic fluorescent pseudomonads, and endophytic aerobic endospore forming bacteria (AEFB) isolated from chrysanthemum roots. Treatments with different letters differ significantly at α=0.05.

	$\log_e$(Total Bacteria cfu), g^{-1}root				loge(Pseudomonads cfu), g^{-1}root				$\log_e$(AEFB cfu), g^{-1}root			
	Cycle 1		Cycle 2		Cycle 1		Cycle 2		Cycle 1		Cycle 2	
Treatment	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1. Control	4.26**b**	0.11	2.89**c**	0.07	1.19**b**	0.9	0	0	0.92**b**	0.69	2.25**b**	0.63
2. Soil steamed once	4.24**b**	0.11	3.37**a**	0.08	2.07**a**	0.9	0	0	1.80**b**	0.48	0	0
3. Soil steamed once + PGPR	4.51**a**	0.11	3.39**a**	0.07	0.47**b**	0.9	0	0	4.22**a**	0.69	3.79**a**	0.63
4. Soil steamed twice			3.10**b**	0.07			0	0			0	0

Table 3. Greenhouse assay. BLAST nearest neighbor identification for the fluorescent pseudomonads isolates from chrysanthemum rhizosphere using 16S rRNA. Percentage of total isolates identified to species level.

Treatment	Sampling week	Number of isolates identified	Blast nearest neighbor	Percentage of isolates
Control[A]	5	40	*P. fluorescens*	25.00
			P. monteilii	5.00
			P. putida	70.00
Steam once[A]		40	*P. fluorescens*	92.50
			P. putida	7.50
Steamed once + PGPR[A]		40	*P. fluorescens*	20.00
			P. fulva	5.00
			P. jessenii	12.50
			P. monteilii	2.50
			P. putida	60.00
Control[A]	10	14	*P. fluorescens*	7.14
			P. fulva	7.14
			P. mosselii	14.29
			P. putida	71.43
Steam once[A]		14	*P. fulva*	7.14
			P. monteilii	50.00
			P. putida	42.86
Steamed once + PGPR[A]		14	*P. citronellolis*	14.29
			P. monteilii	14.29
			P. putida	71.43

[A] First cropping cycle

Chapter III. Screening for potentially deleterious traits in fluorescent pseudomonads isolated from steamed soil

Abstract

Steaming soil, a disinfestation method used to manage soil-borne pathogens, has been shown to increase *Pseudomonas* populations. Some rhizosphere fluorescent pseudomonads cause deleterious effects on plants via production of high levels of indole acetic acid (IAA) and hydrogen cyanide (HCN). We wanted to test the hypothesis that pseudomonads isolated from steamed soil will have the functional traits that are being associated with negative effects in plants. *Pseudomonas* spp. were isolated from rhizosphere soil and from inside the roots of chrysanthemum cultivar Golden Kent planted in non-steamed soil, soil steamed once, steamed soil + PGPR and soil steamed twice. Isolates were identified using the 16S rDNA gene, and characterized by measuring functional traits: indole acetic acid (IAA) and cyanide (HCN) production, and also the effect of bacterial seed inoculation on lettuce root length. Additionally we wanted to investigate whether or not those *Pseudomonas* strains were pathogenic by recording the hypersensitive response (HR) on tobacco and the cell wall-degrading enzyme activity (CWDEA). Results indicated that pseudomonads evaluated were producing differential levels of IAA and HCN and therefore there was an association between the *Pseudomonas* species and the traits. Reduction in lettuce root length was found primarily with inoculation of *P. putida*. None of the pseudomonads evaluated elicited a hypersensitive reaction on tobacco leaves, and the majority of them had cell wall-degrading enzyme activity, thus there was not an association between HR and CWDE and *Pseudomonas* species. Although some species produced higher

levels of IAA and HCN, two known attributes of DRB, effects of soil steaming could not be directly linked to these traits because the same species of *Pseudomonas* were isolated from steamed and non-steamed soil yet in different proportion. Steaming the soil did not result in changes in functional traits of the *Pseudomonas* species, but changed the abundances of the pseudomonads groups that could result in an increased accumulation of bacterially-produced phytotoxic metabolites. Changes in functional traits are specific to bacterial strain levels, implying that within the same *Pseudomonas* species there were differential responses in the functional traits. Overall, the magnitude of the effect of soil steaming is related more to the abundance and composition of the pseudomonad community than the specific functional traits evaluated in this study of any individual *Pseudomonas* species.

Introduction

The rhizosphere is a complex environment shaped by the roots of plants and is the habitat for a large number of microbial groups. Although all of these groups are part of the highly diverse biological web that metabolizes the nutrients exuded by roots (Kluepfel, 1993), only a minority of rhizosphere microbes have been examined thoroughly. Among microorganisms, rhizobacteria have received special attention because of their beneficial traits (Compant et al., 2010). Several bacterial isolates are used as biological control agents or plant growth promoters (PGPR) (Raaijmakers et al., 2009). However, the plant-rhizobacteria interaction does not always result in plant health enhancement, as some of these rhizobacteria have also been reported as deleterious and have negative effects on plant health (Kremer, 2007).

The definition of deleterious rhizobacteria (DRB) initially emerged parallel to the concept of beneficial rhizobacteria as a way to explain possible mechanisms of action of the latter to promote growth. It was stated that PGPR must be able to colonize the root system and

compete with DRB in soil environment, resulting in root growth enhancement. PGPR and DRB are both described as non-infective rhizobacteria, because they do not parasitize or invade vascular tissues, but are aggressive root colonizers able to antagonize other microorganisms in this habitat (Kremer, 2007). Differences between PGPR and DRB are based on the fact that while beneficial strains promote growth, deleterious rhizobacteria inhibit root and overall plant growth.

The positive effects of PGPR on plants are thought to occur through more than one mechanism (Bottini et al., 2004; Compant et al., 2005; Patten and Glick, 2002; Rodríguez and Fraga, 1999). In the same sense, multiple mechanisms are thought to be involved in the inhibition of plant growth by DRB. A number of studies revealed that cyanide, plant growth regulators (auxin), antibiotics or phytotoxic metabolites produced by DRB could be responsible for the detrimental effects in plants (Bakker and Schippers, 1987; Boel et al., 1993; Kremer and Souissi, 2001).

Plants, algae, fungi and bacteria are known to produce HCN (Vennesland et al., 1981). Among bacteria, HCN has been studied extensively in fluorescent pseudomonads, especially *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* (Gallagher and Manoil, 2001). Cyanide produced by *P. fluorescens* strain CHA0 protects plants from root diseases (Voisard et al., 1989). The use of mutants has elucidated that this metabolite accounts for part of the biocontrol capacity of the strain, for example the suppression of tobacco black root rot caused by *Thielaviopsis basicola* (Laville et al., 1998). In *P. aeruginosa,* HCN is a toxic factor that has proved to poison the nematode *Caenorhabditis elegans*, making it one of the virulent factors produced by the bacterium (Gallagher and Manoil, 2001).

In other systems the effect of HCN production by bacteria on the plant has been documented. Alstrom and Burns (1989) conducted a study to examine the production of cyanide as a possible mechanism of plant growth inhibition using *Pseudomonas fluorescens*. One of the *P. fluorescens* strains was an efficient HCN producer and, when inoculated on bean and lettuce, reduced plant dry weight. This growth reduction was related to high levels of cyanide in the rhizosphere compared to non-inoculated plants (Alstrom and Burns, 1989). A bioassay using *Arabidopsis thaliana* as a model showed that cyanide produced by *P. aeruginosa* caused inhibition of root growth. Other interactions in the rhizosphere, such as biofilm formation by *Bacillus subtilis*, were also influenced by the cyanogenesis of the bacterium (Rudrappa et al., 2008). Consequently, there is an effect between HCN production by bacteria, growth of plants and biocontrol of pathogens and the consequences of that interaction for the plant are mediated by the plant host, the soil, and the environment (Defago and Haas, 1990).

Indole acetic acid (IAA) is a phytohormone that can be synthesized by different bacterial groups, including plant beneficial and pathogenic, Gram positive and Gram negative, and nitrogen fixing bacteria (Omer et al., 2004). Two contrasting events can take place in the plant depending on the amount of IAA secreted (Patten and Glick, 2002). Root growth promotion occurs if the IAA level is low i.e. 5 µg/ml (*Acetobacter diazotrophicus* and *Pseudomonas* spp.) or inhibition occurs if the level is high i.e.72 µg/ml (*Pseudomonas putida*) (Barazani and Friedman, 1999). Studies on the effect of IAA produced by bacteria (*P. putida* strain GR1-2) on canola and mung bean showed root elongation in inoculated seeds compared to the non-inoculated control. In contrast, the quantification of IAA in culture supernatants of rhizobacteria including *P. fluorescens* and *P. putida* revealed a correlation between production of IAA and inhibition of root growth of sugar beet (Loper and Schroth, 1986). In general, studies have shown

a correlation between concentration of IAA produced by rhizobacteria and root growth in different plant models, indicating that this metabolite could be beneficial or deleterious for plants and that the effect is concentration-dependent (Sarwar and Kremer, 1995; Xie et al., 1996).

DRB have been isolated from a variety of crops (ferns, potatoes, carrots, citrus, pea, and peach) that exhibited some detrimental effect in their growth, for which no other explanation of symptoms, pathogen presence, or nutritional deficiency, was found (Kloepper et al., 2010; Kremer, 2007).Fluorescent pseudomonads are the most common microbial taxa associated with detrimental effects in plant growth (Schippers et al., 1987). For example, fern distortion syndrome (FDS), a newly described disease (Kloepper et al., 2010) was attributed to changes in pseudomonads. Isolations of microorganisms from symptomatic fronds failed to detect pathogenic fungi, bacteria, or viruses. However, significantly greater populations of endophytic fluorescent pseudomonads were isolated from inside the rhizomes of symptomatic ferns than from asymptomatic ferns in almost all of the locations surveyed.

Management practices, such as tillage, use of pesticides, and monoculture crops have also been linked to the association of DRB with reduced growth (Schippers et al., 1987; Stroo et al., 1988). A study of DRB in a flower monoculture in Colombia found that the main bacterial group associated with chrysanthemum growth reduction in steamed soil was rhizosphere fluorescent pseudomonads (unpublished data-Chapter II). Steaming is used to disinfest the soil prior cultivation and therefore to manage populations of soil-borne pathogens. In addition to the flower monoculture report, tests in a greenhouse pot assay revealed that consecutive steaming reduces the microbial populations after the application is done, but once the soil is planted, fluorescent pseudomonads increase rapidly in the rhizosphere of chrysanthemum.

The overall objective of the studies presented in this chapter was to test the hypothesis that among species of fluorescent pseudomonads previously isolated from steamed and non-steamed soil, there are significant differences in functional traits previously associated with DRB. To test this hypothesis, we established two objectives: 1) to determine if the isolated species differ in their capacities to produce high levels of IAA and HCN, to reduce root length of lettuce in a seedling bioassay, and to cause the hypersensitive reaction in tobacco; 2) to determine if the pseudomonads that had functional traits previously associated with DRB were mainly isolated from the steamed soil.

Materials and Methods

Pseudomonas were isolated from rhizosphere soil and from inside the roots of chrysanthemum cultivar Golden Kent planted in non-steamed soil, steamed soil once, steamed soil + PGPR, and soil steamed twice. Isolates were identified using the 16S rDNA gene, and characterized by measuring functional traits: indole acetic acid (IAA) and cyanide production (HCN), the hypersensitive response (HR), the cell wall-degrading enzyme activity, and the effect on lettuce root length.

Quantification of IAA

All fluorescent pseudomonads isolates were tested for the production of indolacetic acid (IAA) using the protocol suggested by Patten and Glick (2002). Briefly, each isolate was grown in King's B medium at 24 °C for 48 hours. An individual colony of each isolate was transferred to a 50 mL Erlenmeyer containing 10 mL of TSB amended with 5 mM L-tryptophan. Flasks were shaken for 48 h at room temperature. After incubation the cultures were centrifuged for 15 minutes at 3250 rpm. Two mL of the supernatant of each culture was then mixed with 2 mL of Salkoswki's reagent, and the optical density of each one was measured spectrophotometrically at

530 nm. The concentration of IAA was estimated by comparison with a standard curve (Figure 1).

HCN production

Each isolate of fluorescent pseudomonads was grown in King's B amended with 4.4 g/L of glycine. The plates were covered with sterile filter paper impregnated with 0.5% picric acid, 2% sodium carbonate, sealed with parafilm and incubated for 96 h. Change in color of the filter paper to brown indicates HCN production (Figure 2) (Bakker and Schippers, 1987).

Hypersensitive reaction (HR)

Bacterial suspensions (10^6 cells per ml) were prepared with pseudomonads isolated from the rhizosphere of chrysanthemum at every planting cycle. Inoculation on tobacco leaves was done by pressing a syringe containing bacterial suspension against the leaf, followed by the incubation of the tobacco plant in a moisture chamber. Distilled water was used as a negative control. Positive reactions were evaluated 24 and 48 hours after inoculation by the presence of dry and necrotic areas (Goszczynska et al., 2000).

Potato slice bioassay

A bioassay involving potato slices was used to screen for production and secretion of plant cell wall-degrading enzymes (Dong Y-H et al, 2004). Tubers of potato (*Solanum tuberosum* L.) were washed with tap water, dried on a paper towel, and then sliced evenly in ~5 mm sections. The cut surface of the potato slice was inoculated with 100 µl of the pseudomonad suspension at a concentration of 10^6 CFU/ml. Sterilized water was used as a control. All potato slices were placed in covered petri dishes and incubated at 28°C for 48 hours. The presence of a maceration area was recorded as positive for enzyme production (Figure 3).

Lactuca sativa seedlings bioassay

The effect of pseudomonads isolates on the root growth of pre-germinated *L. sativa* was done using the procedure described by Li and Kremer (2000). Briefly, seeds were surface sterilized and germinated overnight on water agar. Pre-germinated seeds with uniform radicals were transferred to water agar plates (5 seeds per plate). Fluorescent *Pseudomonas* isolates were grown in 50 % King's B for 48 hours, re-suspended in sterile distilled water, and 10 µl of the bacterial suspension containing 10^6 CFU/ml was inoculated on each seedling. Control received only sterile water. Four replicates were used for each isolate. Plates were incubated for 6 days in a growth chamber and radical length was then measured (Figure 4).

Statistical analysis

Data from the screening for production of IAA, HCN, cell wall-degrading enzymes and the HR were analyzed using categorical data analysis in SAS version 9.2. Categorical analysis was used because the data were presented in tabular form instead of intervals. The Fisher's exact test was used to detect general associations as an alternative to the null hypothesis between functional traits and pseudomonads species.

The proc GLM procedure was used to analyze the lettuce bioassay response data, because these data were not categorical. We first evaluated distributional assumption using the student panel graphs available in SAS_ Proc GLIMMIX. Since the normality assumption was warranted no data transformation was performed. The equal variance assumption was not fulfilled for root length, and then this response variable was modeled using the group option to create homogeneous variance groups. Dunnett's option was used to assess the differences between treatments and the untreated control. Actual adjusted P-values are presented in the table.

Results

The BLAST analysis of the sequences of the 16S rDNA gene fragment of the fluorescent pseudomonads isolated from the rhizosphere of chrysanthemum revealed a total of seven different species (*Pseudomonas citronellolis*, *P. fluorescens, P. fulva*, *P. jessenii*, *P .monteilii*, *P. mosselii* and, *P.putida*). A number of strains of each species were evaluated for IAA and HCN production, HR response, cell wall-degrading enzyme activity, and their effect on *Lactuca sativa* root length (Figure 5).

IAA production

In the presence of 200 µg/ml of tryptophan all the fluorescent pseudomonads produced IAA (Table 1). The pseudomonad which produced the lowest amount of IAA was *P. putida,* isolated from non-steamed soil (17.38 µg/ml/OD $_{600}$ nm) and the pseudomonad which produced the highest amount of IAA was *P. putida,* isolated from the soil steamed once (141.69 µg/ml/OD $_{600}$ nm) (Appendix 3). For statistical analysis, the level of IAA produced by pseudomonads was classified as low (<60 µg/ml/OD $_{600}$ nm) or high (>60 µg/ml/OD $_{600}$ nm). The categorical data analysis for the IAA functional trait showed a significant association among *Pseudomonas* species and IAA level (P = 0.0291). Greater numbers of IAA producers were found among *P. citronellolis*. An equal ratio of high and low producers was found in *P. putida*. The level of production of the remaining five pseudomonads species belonged to the low level (Table 1). Because *P. putida* was one of the species most frequently found among all treatments and changed proportionally to steam treatment application, a categorical analysis was performed to establish if there was an association between treatment and IAA production for *P. putida*. Results of this analysis showed no association between level of IAA production and treatment. For each treatment *P. putida* low and high IAA producers were found in equal percentages (Table 2).

HCN production

The analysis of the HCN production in the seven species of pseudomonads isolated from the rhizosphere revealed an association between production and species (P-value= 0.00001). Eighty-nine percent of *P. fluorescens* were HCN producers, while in the other species few were found to be HCN producers (Table 1).

Hypersensitive reaction (HR)

The hypertensive reaction was evaluated on one-month old tobacco plants (*Nicotiana tabacum*) in the third open leaf from the top to the bottom. Results of this evaluation showed that none of the fluorescent pseudomonads caused a necrotic area on the leaf 24 hrs after inoculation at 1x 10^6 cfu/ml (Figure 6). However all fluorescent pseudomonads produced a different degree of chlorosis in the surroundings of the inoculation spot. The chlorosis was more severe 48 hours after inoculation which was especially observed with *P. citronellolis*.

Potato slice bioassay

The results of the potato assay, used as an indicator of the presence of cell-wall degrading enzymes, showed that there is no association between pseudomonas species and this trait. However, it is remarkable that a high percentage of the *P. putida* exhibited this enzyme activity (Table 1).

***Lactuca sativa* seedlings bioassay**

The inoculation of fluorescent pseudomonads on the lettuce roots indicated that there is an effect on root growth. The root length of the lettuce inoculated with *P. putida* or *P. mosselii* was significantly less than that of the untreated control (Table 3). Further we analyzed whether or not the steam treatment had an effect on the reduction of root growth by *P. putida.* Results of this analysis indicated that *P. putida* isolated from the steamed soil caused significantly greater

reduction in root length of lettuce, compared to *P. putida* isolated from the non-steamed soil (Table 4).

Functional traits of endophyte fluorescent pseudomonads

Three different species of fluorescent pseudomonads isolated from chrysanthemum root tissue (*P. citronellolis*, *P. fluorescens, and*, *P. putida*) were found as a result of the 16S rDNA gene phylogenetic analysis. A similar analysis of functional traits was performed as for the species isolated from the rhizosphere. The only association found among functional traits and endophyte fluorescent pseudomonas species was for HCN production. A greater percentage of HCN producers among the endophytes corresponded to *P. fluorescens* followed by *P. putida* (Table 5). The results of the lettuce bioassay showed that *P. fluorescens* and *P. putida* had a significant reduction in root length compared to the untreated control (Table 6). Similarly root length was significantly shorter in steamed soil with *P. putida* than from roots of non-steamed soil (Table 7).

Discussion

The overall aim of this study was to characterize fluorescent pseudomonads strains for functional traits that were previously found to be associated with DRB in plants. In addition, we evaluated the cell wall-degrading enzyme activity (CWDEA) and hypertensive reaction (HR) to inquire if the pseudomonas species had virulence factors. We hypothesized that pseudomonad species would have differences in their capacities to produce high levels of IAA and HCN, and to reduce root length of lettuce, and that those species were present mainly in steamed soil. We found that the pseudomonads evaluated produced different levels of IAA and HCN; therefore, there was an association between those two functional traits and pseudomonas species. The majority of the pseudomonas species produced cell wall-degrading enzymes, none of them had a

positive hypertensive reaction in tobacco, and thus CWDEA and HR were not associated to pseudomonas species. Although some species produced higher levels of IAA and HCN, two known attributes of DRB, effects of soil steaming could not be directly linked to these traits because the same species of *Pseudomonas* were isolated from steamed and non-steamed soil yet in different proportion (Figure 5).

All *Pseudomonas* sp. isolated from the rhizosphere and roots were IAA producers. High and low level IAA production was found among and within species. Of the rhizosphere *Pseudomonas* strains, *P. citronellolis* had the greatest relative number of high IAA level producers. *Pseudomonas citronellolis*, originally isolated from soils in north Virginia, has also been found in the rhizosphere of soybean and in bulk soil conducive to tobacco black root rot disease (Kyselkova et al., 2009). *Pseudomonas citronellolis* has been widely studied for its capacity to degrade petroleum hydrocarbons in contaminated soils. In addition to this bioremediation properties *P. citronellolis* isolated from the rhizosphere of soybean has been studied for potential as plant growth promoter (Bhattacharya et al., 2003; Kuklinsky-Sobral et al., 2004; Seubert, 1959). As reported in the soybean study by Kuklinsky-Sobral et al. (2004), we found that *P. citronellolis* produces IAA but in contrast to their growth promotion results in soybean, inoculation of this strain on lettuce seedlings had a significantly reduction of root growth. *Pseudomomas citronellolis* was predominantly found in the soil that was re-steamed, indicating that for this particular species, soil steam treatment changes not only the abundance of pseudomonas in the rhizosphere as previously found, but may also select for high IAA producers. *Pseudomomas citronellolis* was also found as an endophyte in the roots of chrysanthemum plants. All the isolates were high IAA producers, but they did not have any

effect on lettuce root length. This may be an example of two ecotypes, two strains which have homology in the 16S ribosomal gene, yet are ecologically distinct (Konstantinidis et al., 2006).

It is interesting that, overall *P. putida* had a significant reduction in lettuce root length. Additionally, the detailed analysis done on *P. putida* showed that strains from non-steamed, steamed and steamed soil +PGPR had a differential yet reducing effect on root length (Table 4). The *P. putida* strains isolated from steamed soil had the lowest root length values, and they were significantly different compared to the non-steamed soil. These findings suggest that *P. putida* as reported in other models (Berggren et al., 2001) has potentially deleterious traits, those traits can cause growth reduction, and the effect is more severe if the isolates come from steamed soil. In our study root growth reduction was not correlated with IAA production of individual strains because similar percentages of high and low IAA producers were found among steam treatments and the control. Similarly *in vitro* HCN production may not be involved in lettuce growth reduction by *P. putida* because the producers were found in low percentages. These findings show that steaming the soil changes the proportion of this particular species in the rhizosphere of chrysanthemum as reported in the greenhouse pot assay, but it is not associated with changes in metabolite production. Although, it can be argued that the *in vitro* characterization of the functional traits does not imply that those metabolites are going to be produced similarly by the bacteria in the rhizosphere, our results showed that the species composition of the pseudomonas group and their abundance is related to soil steaming. Therefore changes in abundance could affect the synthesis of bioactive bacterial metabolites that impact plant health.

Our results also suggest that depending on the bacterial strain and the environment from where that particular strain was obtained, some metabolites may play a more relevant role in deleterious effects on plants or that the effects on plant growth can be indirect, by influencing

other microorganisms in the rhizosphere. An indirect effect of DRB was reported on the initial infection of pea by *Rhizobium leguminosarum*. Berggren et al (2005) found that *P. putida* strain A313 was deleterious in pea at concentrations lower than 10^7 cfu and that when it was co-inoculated with *R. leguminosarum,* pea nitrogen content in shoots and roots, and size of nodules were significantly reduced (Berggren et al., 2005). In our model system this interaction is not relevant because of the plant model system used (chrysanthemum); however when studying deleterious effects of rhizobacteria on plants, it is important not only to explore for the effect of their metabolites on the plants but also on other plant associated microorganisms (Rudrappa et al., 2008).

When chrysanthemum was inoculated with four PGPR strains and then planted in steamed soil *P. putida* and other species belonging to the same group were found (*Pseudomonas fulva*, *Pseudomonas jessenii*, *Pseudomonas monteilii*, and *Pseudomonas mosselii*). Characterization of the functional traits of these pseudomonad species exhibited variability for HCN production and their effect on lettuce root length. Without considering *P. putida* , the remaining species were low IAA producers. Among them *P. monteilii* and *P. mosselii* caused a reduction on root length. Overall, the effect of application of PGPR in combination with soil steaming did not modify the in vitro functional traits of the rhizosphere *Pseudomonas* group.

Most of the endophyte pseudomonads were high IAA producers but there was no association between this trait and rybotypes. The only association found was between HCN and species. All *Pseudomonas fluorescens* strains were HCN producers. Additionally, *P. fluorescens* and *P putida* had a significant reduction in lettuce root growth. As found for rhizosphere *P putida*, endophyte *P. putida* strains obtained from steamed soil had the lowest lettuce root length compared to the isolates from the non-steamed soil. Again, *P. putida* inoculation effect on root

growth was not related to IAA level, but in the case of *P. fluorescens* root growth reduction could be HCN related.

The hypersensitive reaction (HR) is a defense reaction of plants that can be triggered by bacterial, fungal, viral, or nematode pathogens. The response is characterized by death of plant cells in the inoculated area (Atkinson et al., 1985; Klement and Goodman, 1967). The model system for studying this response has been tobacco. All the rhizosphere or endophyte pseudomonads species evaluated for HR had a negative response. Interestingly, all the isolates produced a different level of chlorosis surrounding the inoculation area of the tobacco leaf. HR results showed that although the *Pseudomonas* species were not pathogenic, they can produce certain metabolites that result in the chlorotic symptoms observed. Chlorosis-inducing toxins have been reported in pathogenic species of pseudomonads. A study in tomato using different pathovars of *P.*syringae revealed that the bacterium produces a chlorosis-inducing factor called coronatin (Uppalapati et al., 2010). However, similar toxins have not yet been associated with deleterious pseudomonas.

Many phytopathogenic microorganisms secrete cell wall-degrading enzymes to penetrate the plants barrier made of polysaccharides (Juge, 2006). For *Pectobacterium carotovorum*, cell wall-degrading enzymes are one of the major virulence factors of soft rot (Andresen et al., 2007). We used the potato slice assay to detect the presence of these enzymes in the pseudomonads. Although there was not an association between pseudomonas species and the capacity to macerate potato tuber tissue, we found that most of the *P. putida* strains evaluated had this capacity, and this group was more apt to change when the soil was steamed. HR and CWDEA can be used to distinguish between pathogenic and non-pathogenic microorganisms. Based on the evaluation of these traits in the pseudomonads strains none of them were pathogenic,

suggesting initially that steaming does not lead to pathogenecity. However, an important percentage of pseudomonads produced a differential degree of chlorosis on tobacco and was able to macerate the potato tuber tissue suggesting that although there are not pathogens they are producing toxins or enzymes that could be virulent traits.

There are traits that characterize the pseudomonas species that are associated with deleterious effects in plants. We found a specific association for a particular rybotype and a functional trait but also variation in the functional response with different strains of the same species. Further, we found that there is not a functional trait that distinguishes the pseudomonas isolated from the steamed and non-steamed soil. Consequently the effect of soil steaming on the pseudomonads group is strain-dependent and not species dependant. Identifying the bacteria to using 16S is important; however the physiology of the bacterium and the expression of functional traits are also important when the abundances have been modified. Without a doubt, steaming not only influences the relative abundance pseudomonas species, but also changes their absolute abundance.

In general, production of the above-mentioned compounds will help to better characterize the deleterious effects of rhizobacteria and to find under which conditions those effects are expressed or over-expressed to result in plant growth reduction. The factors that distinguishes DRB from PGPR perhaps results from the plant-microbe interactions mediated by the environment in which the interaction takes place. Suggesting that production of a less than critical levels of metabolites makes rhizobacteria beneficial for certain plant species, but deleterious when production of metabolites exceeds critical levels or alternatively when the abundance of the population changes, the amount of the metabolite can get to critical levels, injurious to the plant health.

In our study, the magnitude of the effect caused by soil steaming on the *Pseudomonas* group relies more heavily on changes on microbial abundance and species composition than on functional traits. We found that there were some minimal associations between functional traits and species of *Pseudomonas*, no relation between in vitro-expressed functional traits and steam treatment, and a negative effect on lettuce root growth for specific pseudomonas strains coming from steamed soil.

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Table 1. Evaluation of IAA, HCN, Hypersentive reaction (HR) and cell wall-degrading enzyme activity (CWDEA) of fluorescent pseudomonads isolated from the rhizosphere of chrysanthemum.

Pseudomonas species	IAA		HCN producers	HR	CWDEA	Total strains screened
	High	low				
P. citronellolis	67%	33%	0%	0%	33%	6
P. fluorescens	0%	100%	89%	0%	56%	9
P. fulva	25%	75%	50%	0%	75%	4
P. jessenii	0%	100%	0%	0%	100%	1
P. monteilii	50%	50%	0%	0%	75%	4
P. mosselii	0%	100%	100%	0%	100%	2
P. putida	49%	51%	11%	0%	76%	37
Pr <=P	0.0291 [A]		<0.00001 [A]	ND	0.3746 [A]	63

[A] Contingency table of categorical data analysis showing the probability of association among functional trait and species using Fisher's exact test.
ND = not determined because none of the fluorescent pseudomonads strains screened had a hypersensitive reaction.

Table 2. Evaluation of IAA production of *Pseudomonas putida* isolated from the rhizosphere of chrysanthemum.

Treatment	IAA production		Total strains screened
	high	low	
Non-steamed soil	50%	50%	12
Soil steamed once	49%	51%	9
Soil steamed once + PGPR	50%	50%	16
Pr <=P	1.00[A]		

[A] Contingency table of categorical data analysis showing the probability of association among functional trait and treatment using Fisher's exact test.

Table 3. Least square means, degrees of freedom for error (df), standard error, and the probability of the difference between the untreated control (Dunnet's test) for fluorescent pseudomonads isolated from the rhizosphere of chrysanthemum on lettuce root length.

	Lettuce root length (cm)			
Pseudomonas species	Means	df	Standard error	Dunnet's P
P. citronellolis	2.689	312	0.16	<0.0001
P. fluorescens	2.961	312	0.15	0.006
P. fulva	2.919	312	0.2	0.049
P. jessenii	2.935	312	0.37	0.549
P. monteilii	2.977	312	0.18	0.046
P. mosselii	2.518	312	0.16	<0.0001
P. putida	2.791	312	0.14	<0.0001
Untreated control	3.469			

Table 4. Least square means, standard error, degrees of freedom for error (df), and the probability of the difference between the untreated control (Dunnet's test) for *Pseudomonas putida* isolated from the rhizosphere of chrysanthemum on lettuce root length.

	Lettuce root length (cm)			
Treatment	Means	df	Standard error	Dunnet's P
Non-steamed soil	3.002			
Soil steamed once	2.629	187	0.060	<0.00391
Soil steamed once + PGPR	2.822	187	0.084	0.2816

Table 5. Evaluation of IAA, HCN, Hypersentive reaction (HR), and cell wall-degrading enzyme activity (CWDEA) of endophytic fluorescent pseudomonads isolated from chrysanthemum.

Pseudomonas species	IAA		HCN producers	HR	CWDEA	Total species screened
	High	low				
Pseudomonas citronellolis	100%	0%	17%	0	50%	6
Pseudomonas fluorescens	71%	29%	100%	0	72%	7
Pseudomonas putida	72%	28%	64%	0	82%	11
Pr <=P	0.537[A]		0.062 [A]	ND	0.4446 [A]	24

[A] Contingency table of categorical data analysis showing the probability of association among functional trait and species using Fisher's exact test.
ND = not determined because none of the fluorescent pseudomonads strains screened had a hypersensitive reaction.

Table 6. Least square means, degrees of freedom for error (df), standard error, and the probability of the difference between the untreated control (Dunnet's test) for endophytic fluorescent pseudomonads isolated from chrysanthemum on lettuce root length.

	Lettuce root length (cm)			
Pseudomonas species	Means	df	Standard error	Dunnet's P
P. citronellolis	3.111	96	0.2	0.139
P. fluorescens	2.744	96	0.19	0.0006
P. putida	2.578	96	0.19	<0.0001
Untreated control	3.501			

Table 7. Least square means, standard error, degrees of freedom for error (df), and the probability of the difference between the untreated control (Dunnet's test) for endophytic *Pseudomonas putida* isolated from chrysanthemum on lettuce root length.

	Lettuce root length (cm)			
Treatment	Means	df	Standard error	Dunnet's P
Non-steamed soil	3.2			
Soil steamed once	2.2	41	0.169	<0.00001
Soil steamed once + PGPR	1.95	41	0.165	<0.00001

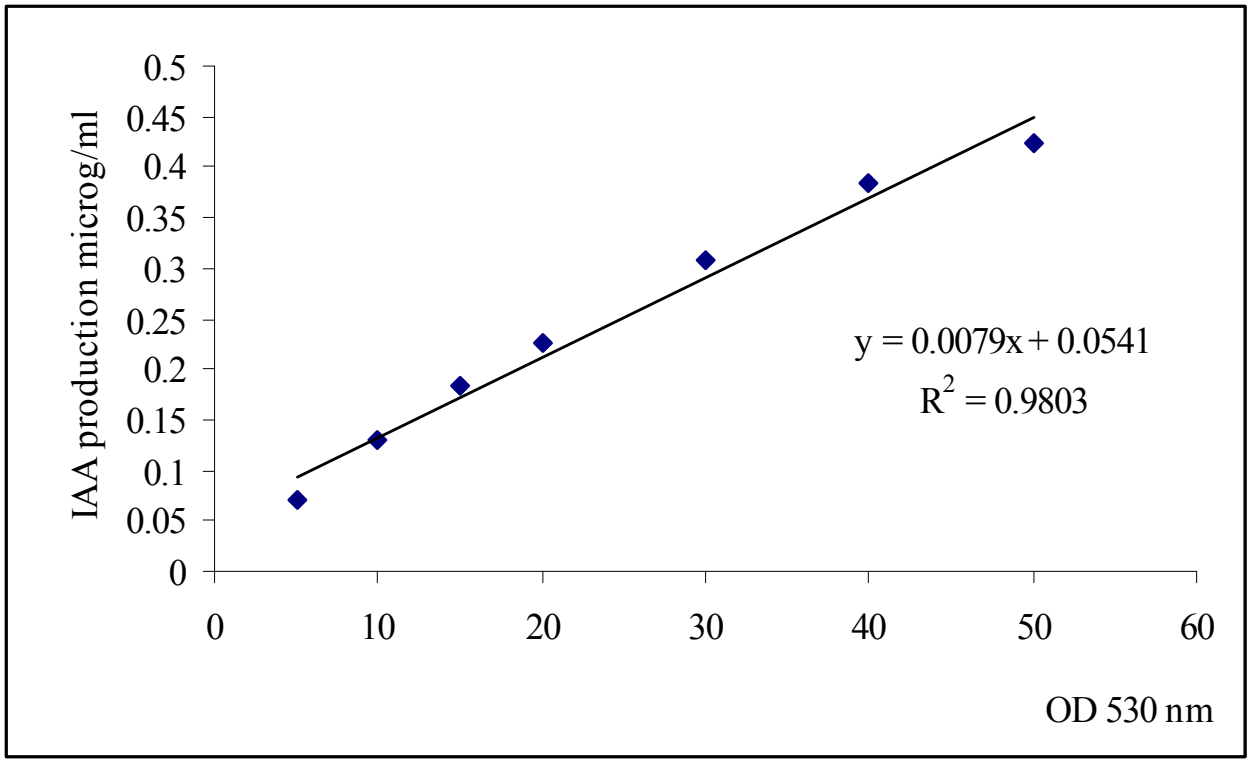


Figure 1. Standard curve to determine the IAA production of fluorescent pseudomonads

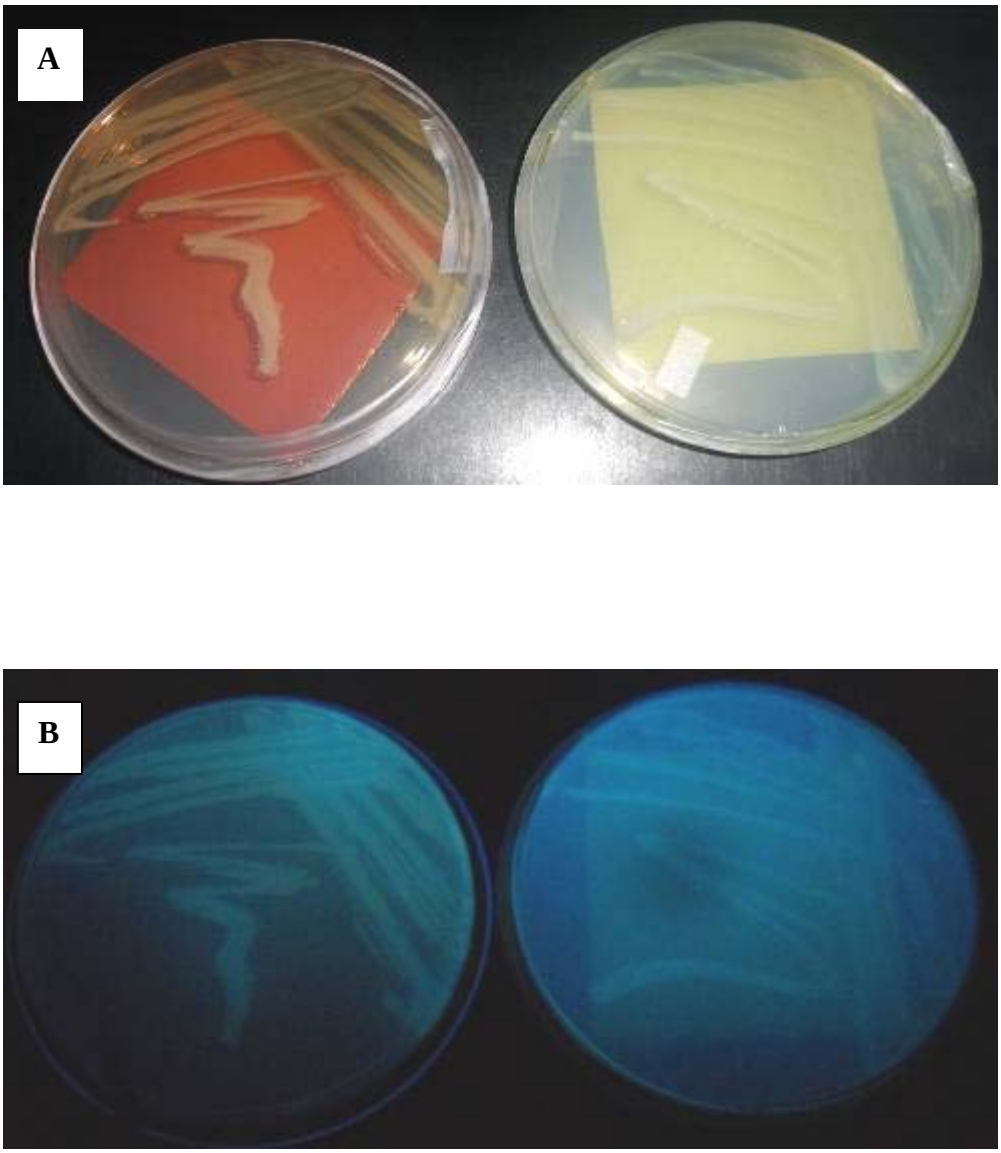


Figure 2. Evaluation of HCN production of fluorescent pseudomonads isolated from the rhizosphere of chrysanthemum. A. left plate HCN producer, right plate HCN non-producer. B left plate HCN producer, right plate HCN non-producer under UV light

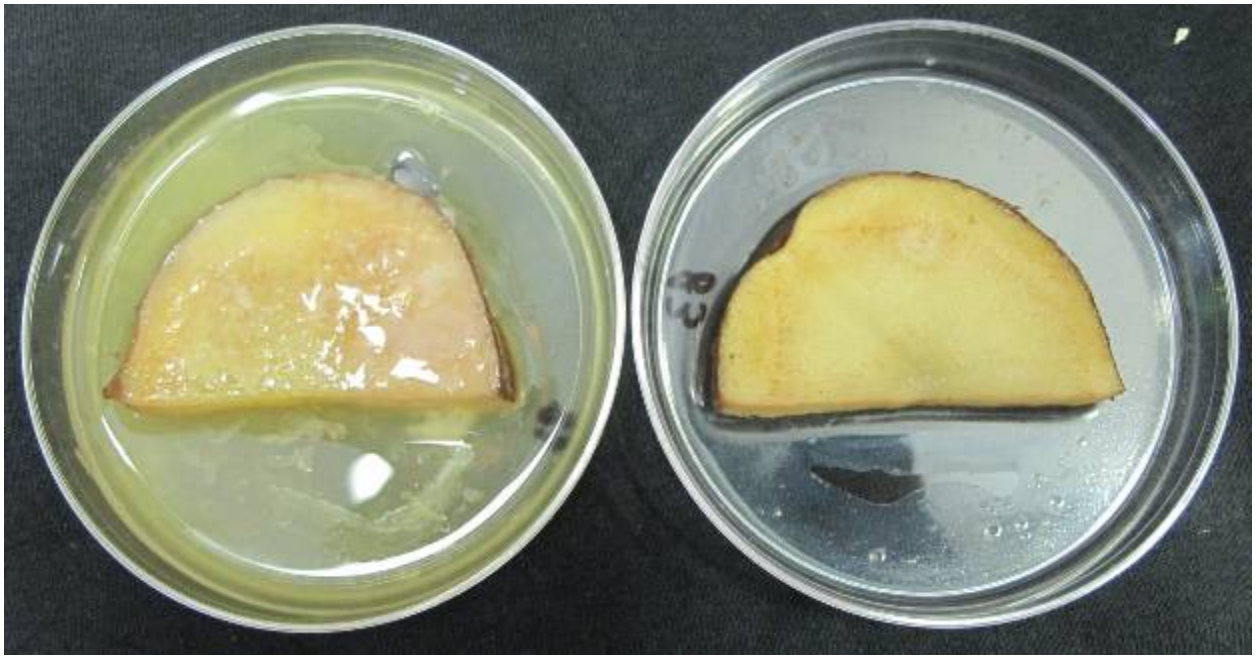
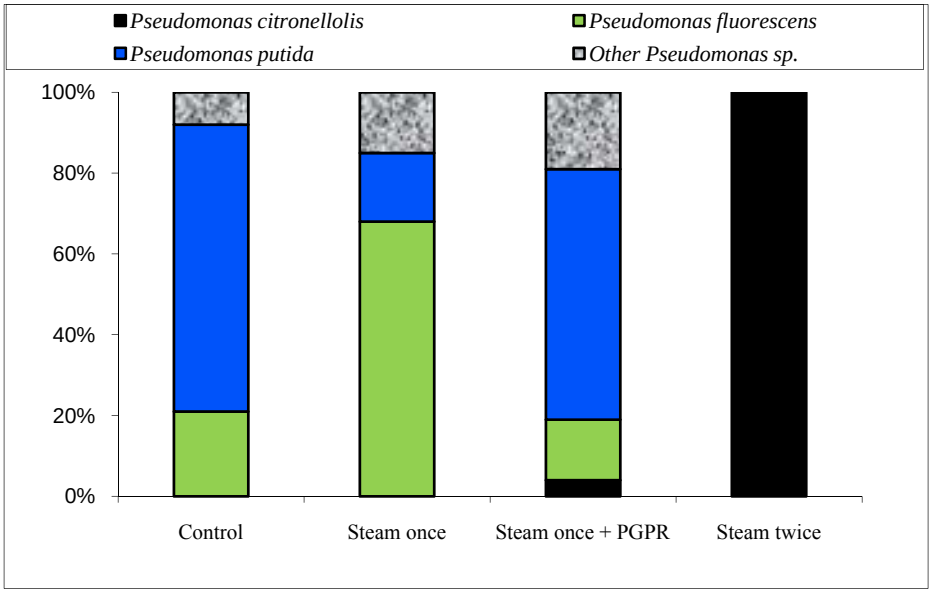


Figure 3. The effect of fluorescent pseudomonads (left plate) on potato 72 hours after inoculation. Right plate water control.

Figure 4. The effect of fluorescent pseudomonads (left plate) on elongation of roots of lettuce seedlings five days after transplanting. Right plate control

a)



b)

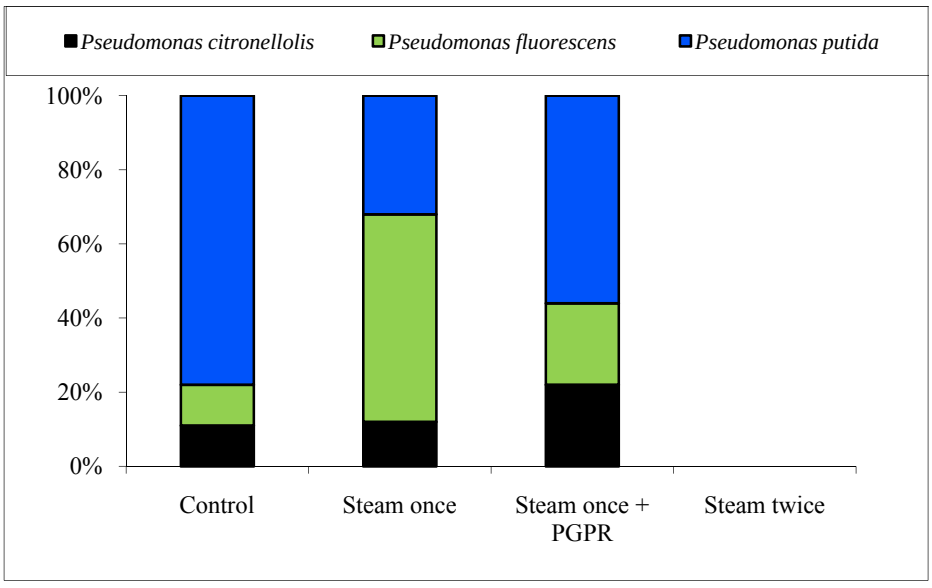


Figure 5. Percentage of fluorescent pseudomonads isolated from chrysanthemum identified to species level. a) Rhizosphere b) Endophytes

a)

b)

Figure 6. The effect of fluorescent pseudomonads on tobacco leaves a) 24 hours after inoculation and b) 48hours after inoculation

Chapter IV. Analysis of bacterial communities via DGGE after steaming of chrysanthemum soil

Abstract

Steaming of soil to control soil-borne pathogens is a common practice in high-value green-house and row crops. In the past decade, reports about the effect of soil steaming on the soil microbial community have been published. Overall, those studies have documented the impact of this practice on nitrifying and denitrifying bacteria, total bacterial abundance, microbial activity, and bacterial community structure. The majority of studies focused on the effect in bulk soil, while the remaining few studies focused on soil steamed once. In the current study we report on the effects of consecutive steaming of soil on the eubacterial community and also on two specific bacterial groups, *Pseudomonas* and *Bacillus,* members of which are common pathogens, growth promoters, biological control agents or deleterious bacteria. The overall aim was to determine the effect of consecutive steam application on the microbial community structure in the rhizosphere of chrysanthemum in terms of the aforementioned specific groups. Soil samples were collected from chrysanthemum plants cultivated in soil that was steamed once, steamed once + PGPR, steamed twice, and also from a non-steamed soil. To assess the effect of consecutive steaming on the soil bacterial communities DGGE, a molecular technique based on the 16S rDNA was used. Comparing 16S- fingerprints showed that no significant differences were found among non-steamed soil, steam soil once and steam soil twice for eubacterial community structure. In other words steaming does not shape the whole rhizosphere community of chrysanthemum plants, what it does is the inoculation of their roots

with PGPR. When *Pseudomonas* and *Bacillus* specific primers were used, relevant differences were found among the rhizosphere communities of the non-steamed and steamed soil.

Introduction

Soil steaming is a disinfestation practice that utilizes heat to reduce soil borne pathogens in greenhouses and open fields. It is commonly used in the commercial flower industry during the preparation of the planting beds because soil-borne pathogens represent one of the major problems in this production system (Pizano, 2001). Although the targets of steam application are pathogens and weeds (Awuah and Lorbeer, 1991; Melander and Jorgensen, 2005) heating the soil also affects beneficial microbiota (Chen et al., 1991; Yamamoto et al., 2008) due to the damage that heat may induce in cell membranes, ribosomes, and proteins (Russell, 1999).

The effect of soil steaming on bacterial groups has been studied in bulk and rhizosphere soil. Roux-Michollet et al (2008) found that the steaming of bulk soil immediately reduced abundance and activity of heterotrophic, nitrifying and denitrifying bacteria. Although, populations of these groups were recovered by the end of the experiment, nitrifying enzymatic activity was reduced to below detectable levels in steamed soil. Additionally, shifts in eubacterial community composition during the duration of the experiment (60 days) were recorded (Roux-Michollet et al., 2008). Another study conducted in bulk soil showed that soil steaming resulted in increased levels of NH_4-N and inhibition of nitrification. Compared to fumigation with methyl bromide and chloropicrin, steaming has strong effects on the microbial biomass and activity, and severely alters the soil community structure (Yamamoto et al., 2008). Similarly, reductions in ammonium and nitrite oxidizing bacteria were found in the rhizosphere of tomato planted in steamed soil (Tanaka et al., 2003). Effects of soil steaming can also result in changes in other bacterial taxa in the rhizosphere of plants. A study conducted in theColombian flower industry

under natural field conditions showed significantly increased populations of fluorescent pseudomonads in the rhizosphere of chrysanthemum after soil steam application (Ramirez et al., unpublished data).

In addition to changes in the bacterial communities, growth promotion and growth reduction in plants have been attributed to the steaming of soil. Tanaka et al (2003) reported growth promotion in tomato. Plants grown in steamed soil had longer stems and higher leaf area in the initial stages of a 4 month experiment. Although positive effects were found in tomato growth, steaming also resulted in reduction of important soil microbial communities and their associated functions. Growth promotion observed in other plants cultivated in steamed soil has been attributed to different factors, including enhanced decomposition and mineralization of organic matter, increase in available N, K, and P, and changes in NH_4^+/NO_3^- (Chen et al., 1991). Soil steaming has also been associated with growth reduction in other crops and was attributed to toxic effects of increases in Mn^{2+} and NO^{2-} in the soil (Chen et al., 1991).

Several studies have examined the effects of steam on soil microbial communities and plant growth. However, there are no reports examining the effects of repetitive steam application on indigenous soil microbial communities and on plants. Such studies are needed because flower growers typically use repetitive soil steaming as a cultural practice in intensive production of cut flowers. Hence, it is important to determine the effects of repetitive steaming on soil microbes. In this study we used a cultivation independent technique, the denaturing gradient gel electrophoresis (DGGE) to study microbial communities in the rhizosphere of chrysanthemum planted in steamed and non-teamed soil. DGGE was selected because it has been successfully used to study microbial communities in different habitats including soil (Milling et al., 2005; Okubo and Sugiyama, 2009; Vainio and Hantula, 2000; Zhong et al., 2007). The overall aim of

this part of the dissertation was to determine the effect of consecutive steam application on the microbial community structure in the rhizosphere of chrysanthemum, in terms of specific groups (*Pseudomonas* spp. and *Bacillus* spp.). The overall hypothesis was that repeated soil steaming will change the bacterial community structure. An additional hypothesis was that soil steaming alters the structure of fluorescent *Pseudomonas* group in the soil.

Experimental approach

For analysis of rhizosphere bacterial communities in the chrysanthemum soil, total DNA was extracted for each treatment of two consecutive planting cycles of a pot assay set up under greenhouse conditions. In the first cycle treatments included steamed soil, steamed soil + PGPR, and non-steamed soil. For the second cycle, half of the steamed soil was re-steamed, adding a fourth treatment.

Soil DNA extraction and rDNA amplification

Total community DNA was extracted from the rhizosphere soil with the Ultraclean™ Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). DNA extraction was done according to the manufacturer's protocol. DNA quality and quantity was assessed by measuring the concentration using the NanoDrop 100 Spectrophotometer (Thermo Scientific, USA) and also was verified with 1.5% agarose gel. DNA was stored at – 20 °C until further use.

Bacterial 16S rDNA gene fragments were amplified using the primers 968-GC (5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and 1401 R (5'- CGG TGT GTA CAA GGC CCG GGA ACG-3') (Smalla et al., 2001). The reaction mixture (25 µl) consisted of 2.5 µl of template DNA (1-5 ng) and 22.5 µl aliquot PCR mix. The PCR mix was as follows: 12.5 µl of EconoTaq® plus green 2X Master Mix (Lucigen Corporation, Middleton, WI, USA), 0.25 µL of each primer (20 µM, Invitrogen

Corporation, CA, USA) and 9.5 µL PCR certified water (Teknova, CA, USA). Thermocycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94 °C for 1 min, 53°C for 1 min and 72°C for 2 min, and a final elongation at 72°C for 10 min. After the cycle program was performed PCR products were checked by electrophoresis in 1.5 % (w/v) agarose gel followed by ethidium bromide staining to confirm product size and negative control.

For amplification of the *Pseudomonas* the primer system 311Ps (5'- CTG GTC TGA GAG GAT GAT CAGT -3') and R1459Ps (5'- AAT CAC TCC GTG GTA ACC GT -3') described by Milling et al, (2004) were used. The thermal cycling was as follows: initial denaturation at 94°C for 7 min, followed by 25 cycles of 94°C for 1 min, 63°C for 2 min, 72°C for 2 min, final extension at 72°C for 10 min. The PCR products (expected sizes about 1100 bp) of the reaction were checked 1.5% agarose gel. For DGGE analysis, the product of the first PCR reaction was used as a template for a second PCR based on the bacterial primers F968 (with GC clamp) and R1401.

For amplification of the *Bacillus* and related taxa the primer system BacF (5'-GGG AAA CCG GGG CTA ATA CCG GAT-3') described by Garbeva, et al (2003) and R1401 universal bacterial 16S was used. The thermal cycling was as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 65°C for 90 s, 72°C for 2 min, final extension at 72°C for 10 min. The PCR products (expected sizes about 1300 bp) of the reaction were checked 1.5% agarose gel. For DGGE analysis, the product of the first PCR reaction was used as a template for a second PCR based on the bacterial primers F968 (with GC clamp) and R1401.

Denaturant gradient gel electrophoresis (DGGE)

The bacterial community analysis was carried out with DGGE analysis for 16S rDNA product using the Dcode® Universal Mutation Detection System (Bio-Rad, Hercules, CA). Three samples for each treatment and cycle combination were run. The standard marker was composed of 16s rDNA products generated from (in order to the migration distance): *Pectobacterium carotovorum*, *Rhizobium radiobacter*, *Variovorax soli*, *Pseudomonas fluorescens*, *Bacillus amyloliquefaciens, Methylobacterium mesophilicum*, and *Nocardiopsis dassonvillei*. Denaturants gradient 0f 50-65% (100% denaturant corresponds to 7 M urea and 40 % (v/v) of deionized formamide) denaturant were prepared. The polyacrylamide gels were prepared by mixing 12 mL of each denaturant solution using a manual gradient delivery system (Bio-Rad, Hercules, CA). The 6% acrylamide gel was polymerized overnight and then 25 μL of PCR products were applied on the gel. The DGGE was performed in 1X TAE at 60 °C and a constant voltage of 80 V for 17 h. After the electrophoresis, the gel was stained with ethidium bromide for 60 minutes and rinsed for 30 minutes with deionized water. The gel was visualized using the UV illumination system AlphaImager (Alpha Innotec Corp., San Leandro, CA).

The DGGE gel image was analyzed with BioNumerics V. 5.0 software program (Applied Maths, Austin, TX). Following conversion, normalization, and background subtraction with mathematical algorithms, levels of similarity between profiles were calculated with the curve based Pearson coefficient. Cluster analysis was performed with the Unweighted Pair Group Method using Arithmetic averages (UPGMA). Multi-Dimensional Scaling (MDS) was completed to compare the clusters generated over different treatments.

Results

Eubacterial 16S rDNA fragments amplified from total community of chrysanthemum rhizosphere were compared running three replicates of each treatment in parallel on one DGGE gel (Figure 1a, Figure 3a). The treatments for the first planting cycle included non-steamed soil, steamed soil, and steamed soil + PGPR. For the second cycle, half of the steamed soil was re-steamed, adding a fourth treatment (soil steamed twice).

For the first planting cycle the rhizosphere fingerprints of total bacterial community showed a high degree of similarity for non-steamed and steamed soil (60%) (Figure 1b). The similarity for steamed soil + PGPR compared to non-steamed and steamed soil profiles was lower (30%) (Figure 1b). Bands specific for non-steamed or steamed soil were not observed. In contrast the steamed soil + PGPR showed fewer bands, some of which were only found in soil from the rhizosphere of chrysanthemum plants that were inoculated with PGPR. Overall there was a high similarity of the DGGE patterns within each treatment for the first planting cycle with one outlier (Figure 2).

For the second planting cycle the DGGE profile of the bacterial communities for all the treatments showed a higher similarity as follows: steamed soil and steamed soil + PGPR profiles had very high (96%) similarity; soil steamed twice had high profile similarity (92%) with steamed soil treatment and steamed soil + PGPR treatment; and the profile of non-steamed soil had high similarity (85%) with steamed soil treatments (Figure 3b). Multi-dimensional scaling (MDS) of the DGGE profiles for each treatment placed them in different clusters. The steamed soil and steamed soil + PGPR clustered together with the exception of one outlier. Soil steamed twice and non-steamed soil each formed separate clusters (Figure 4).

To increase the sensitivity while reducing the complexity of the DGGE patterns, primers specific for *Pseudomonas* and *Bacillus* were used to analyze chrysanthemum rhizosphere communities (Figure 5, Figure 7, and Figure 9). In the first planting cycle the DGGE profile for the *Pseudomonas* group showed two main clusters with 40 % similarity between them (Figure 5b). The first cluster corresponded to the steamed soil treatment and the second cluster to the treatments of non-steamed soil and steamed soil + PGPR. The MSD of the DGGE community profile revealed those two clusters but also the presence of outliers, suggesting variability within the non-steamed soil and steamed soil + PGPR treatments. DGGE community analysis for the *Pseudomonas* group was not performed in the second planting cycle because PCR did not work for those DNA samples under the same condition that was successfully done for the first cycle.

In the first planting cycle the DGGE profile for the *Bacillus* group showed two main clusters (Figure 8). The first cluster included steamed soil and steamed soil + PGPR, and the second cluster was formed with the profile of the non-steamed soil treatment. The DGGE profiles of the *Bacillus* communities from steamed soil and steamed soil + PGPR had 75% similarity, while the cluster formed by these two treatments had 40 % similarity with the profile from non-steamed soil (Figure 7b). In the second planting cycle three main clusters were generated (Figure 10). The first cluster corresponded to steamed soil and steamed soil + PGPR profiles with 90 % similarity between them. The second cluster included the non-steamed soil profile, which had 50 % similarity with the first cluster (steamed soil and steamed soil + PGPR). The last cluster profile (soil steamed twice treatment) had very low (20 %) similarity with the first and second clusters (Figure 9b).

Discussion

The overall aim of this project was to determine the effect of consecutive steam application on the microbial community structure in the rhizosphere of chrysanthemum, in terms of specific microbial groups (*Pseudomonas* spp. and *Bacillus* spp.). We hypothesized that soil steaming changes the whole bacterial community structure when it is applied repeatedly and further modifies the structure of heat-intolerant groups, such as *Pseudomonas*. Results indicated that steaming does not change the overall rhizosphere community structure in chrysanthemum cultivated in soil steamed once or twice, since only slight differences were observed in the profiles of communities from steamed treatments compared to non-steamed soil. However, changes in community structure were recorded for specific bacterial groups, i.e. *Pseudomonas* and *Bacillus*, partially supporting the starting hypothesis.

We evaluated the effect of an abiotic environmental factor, heat in the form of steam, on the rhizosphere microbial communities of chrysanthemum. Interestingly we found that steaming the soil prior to cultivation does not shape the community structure as has also been reported for other disturbances, such as desiccation (Fierer et al., 2003). Contrary, changes in pH another abiotic factor, have been found to predict shifts in soil bacterial communities better than land use practices (Lauber et al., 2008). Analysis of the chemical properties of the steamed soil revealed that steaming does not change the soil pH (Appendix 7), suggesting that a mechanism other than pH drives the changes in bacterial communities in steamed soil. Steaming changed mainly the levels of manganese, an effect previously reported (Sonneveld, 1979). However, the increased level of manganese was not toxic to the chrysanthemum plants or influential of the bacterial community structure, indicating that changes in Mn levels do not drive changes in either plant characteristics or microbe populations.

Bands specific for non-steamed and steamed soil were not observed, indicating that steaming does not affect the relative abundance of dominant bacterial species in the rhizosphere. In contrast, fewer bands were obtained from steamed soil + PGPR suggesting that introducing PGPR into steames soil reduces the complexity of the bacterial community in the rhizosphere. Addition of PGPR changed the overall community structure only when they were first introduced, i.e., changes were evident only in the first planting cycle. For the second planting cycle, when plants were once again inoculated with PGPR, those same changes in the bacterial community were not observed. In fact, the similarity between fingerprint patterns of steamed soil and steamed soil + PGPR was higher, implying that once PGPR were introduced, no further modifications were produced.

The mixture of PGPR consisted of four *Bacillus* strains, *B. pumilus* INR7, *B. amyloliquefasciens* FZB42, *B. amyloliquefasciens* MBI600, *B. amyloliquefasciens* GB03, each applied at a concentration of 1x 10^7 cfu/ml. PGPR strains were selected because individually they were highly antagonistic against fungal pathogens and deleterious fluorescent pseudomonads. Whether or not the observed changes in the soil community structure were related to antibiotic production of the PGPR in the rhizosphere needs to be further investigated. Although changes to the microbial community were found in the profile of soil steamed + PGPR, compared to other groups, those changes were not detrimental for the plant health.

Steaming changed the *Pseudomonas* community, an effect previously reported for some nitrifiers and denitrifiers in soil subjected to steaming (Tanaka *et al*., 2003). Surprisingly, steaming the soil also had an effect on the heat tolerant groups. This suggests that steaming does not modify the bacterial community solely by selecting against heat-intolerant groups. The *Bacillus* community structure was changed due to the soil steaming but this was not due to the

application of PGPR, which are also *Bacillus* sp. Steaming the soil changed the profiles of the *Bacillus* group, while inoculation with PGPR did not, as shown in the dendogram that grouped together the treatments of steamed soil + PGPR and non-steamed soil for both planting cycles (Figure 7, Figure 9). However, in the second planting cycle the similarity of profiles from all treatments was lower for *Bacillus* groups. *Bacillus* species are known to produce spores. Spore formation is initiated by starvation of carbon, nitrogen or phosphorus (Piggot and Coote, 1976). After completion of successive physical and biochemical changes the mature spore cells are metabolically dormant and highly resistant to extreme environmental conditions such as heat or osmotic stress (Antelmann *et al.*, 2000). In the vegetative stage the bacterial cells are metabolically active and use the nutrients available in the medium (Eichenberger, 2007). Soil steaming induces changes in the nutrient availability, allowing vegetative cells of heat tolerant bacteria to actively grow. In this scenario, where food resources are merely supplied, the conditions for starvation are not warranted, giving a disadvantage to the vegetative cells. Consequently the changes in the community profile that steaming produced in the *Bacillus* group of rhizosphere soil can be explained by the sensitivity to heat of *Bacillus* vegetative cells. In addition, spore formation is an energy demanding process and thus bacterial cells are not going to initiate sporulation if the survival of vegetative cells is not being compromised (Eichenberger, 2007). Another possible explanation for the observed changes in the *Bacillus* rhizosphere profile after soil steaming is that elevated temperatures experienced by soil microbes during steaming could cause protein inactivation, leading to lack of spore formation. The process of sporulation takes time and involves gene regulation and expression of over 70 proteins that play a key role in the successive stages of initiation and final formation of a mature spore (Eichenberger, 2007).

Repeated steaming changed the *Bacillus* community, but not the overall bacterial community structure, implying that repetitive steaming will select for or against specific bacterial groups. Due to the complexity of the soil environment, in terms of bacterial diversity and community structure, the molecular analysis of the effects of steaming on the bacterial community should be addressed in terms of specific groups. In fact, specific primers have been designed to look for the effects of disturbances on targeted groups (Costa et al, 2006). The challenge will be to decide which of the many soil bacterial groups will be the focus of the study.

DGGE, the technique chosen to analyze bacterial communities in this study, has both positive attributes and shortcomings which should be taken into account when interpreting the results. While several studies have used DGGE as a useful tool for studying bacterial communities (Costa *et al*., 2006; Frapolli *et al*., 2010; Okubo and Sugiyama, 2009), other studies have documented the multiple complicated aspects that need to be addressed to get results. Once the effective method for DNA extraction is found (Ning *et al.*, 2009), the next challenge consists of reducing the formation of heteroduplex (Rettedal *et al.*, 2010), a molecule which forms when two highly similar sequences anneal to each other rather than to the complementary strands (Muyzer and Smalla, 1998). In DGGE gels, heteroduplex can be seen as faint bands that do not correspond to actual ribotypes, resulting in spurious bands and erroneous results. The GC clamp of the forward primer should be C-rich to decrease the degree of the GC clamp error (Rettedal *et al.*, 2010). The preparation of the polyacrylamide gel should be consistent in terms of purity and freshness of the reagents, temperature and degasification time of the denaturants, to avoid inhibition or partial polymerization which results in different porosity, and therefore, different mobility of the molecules within the gel (Bio-Rad, Bulletin). The duration and voltage of the electrophoresis can also impact the community profiles obtained. Sigler *et al.*, (2004) found that

shorter periods of time at higher voltage will lead to better resolved gels, or in other words, a greater amount of visible bands in the profile. Combs with 25 or 30 wells are available for the Bio-Rad system, however not all lanes can be used because the samples loaded in the outer lanes will not run at the same rate as the inner lanes, resulting in a "smiling" effect. This issue can be solved by adding grease to the spacers before the assembly of the gel sandwich (Brinkhoff, 2001). Perhaps, most importantly, multiple bands can be generated for a single bacterial species, depending on the number of copies of the targeted gene and a single band does not always represent a single bacterial strain (Sekiguchi *et al.*, 2001). Therefore, cloning and sequencing are necessary to determine the actual identity of the bacterial groups. The quality of the image should be very good to be successfully analyzed, and staining with Gelstar instead of ethidium bromide gives brighter bands. Finally, the consistency of the gels is critical to be able to perform gel-to-gel comparisons. The complicated analysis of the images adds to the many variables that contribute to the potential unreliability of this technique. Performing as many steps as possible robotically should minimize the variability inherent in the technique. In addition a web site with a discussion forum (http://ddgehelp.blogspot.com/) exists where DGGE users can continually look for new issues with this technique that makes it as complex as the environment that it is trying to address.

Assuming that the artifacts associated with DGGE affected each treatment similarly, we can conclude that steaming the soil once does not have a major impact on the soil community of the rhizosphere, but does impact to some extent the soil microbial community when reapplied. However, potentially important differences in the communities of specific bacterial groups (*Pseudomonas* and *Bacillus)* are caused by steaming. The exact consequences of these changes

are not known, since the effects of each bacterial species or strain on plant growth varies from one group to the next.

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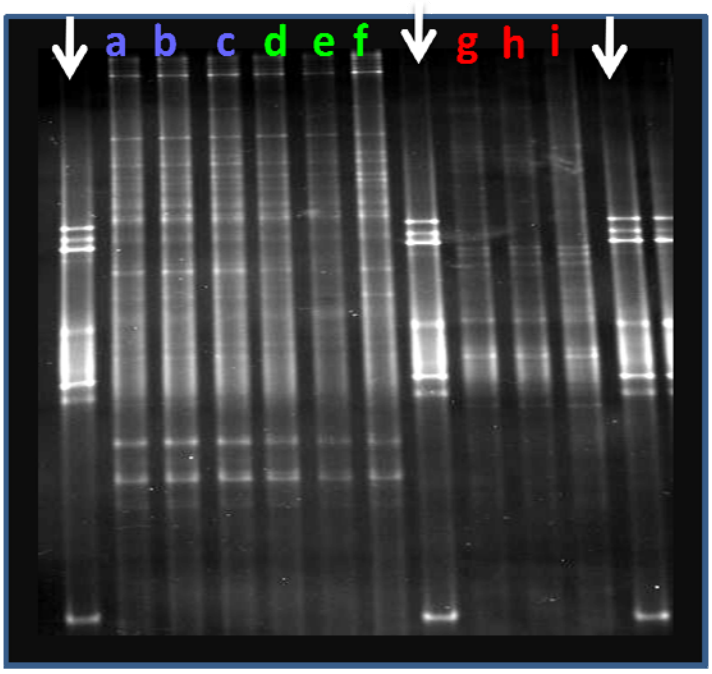
a)



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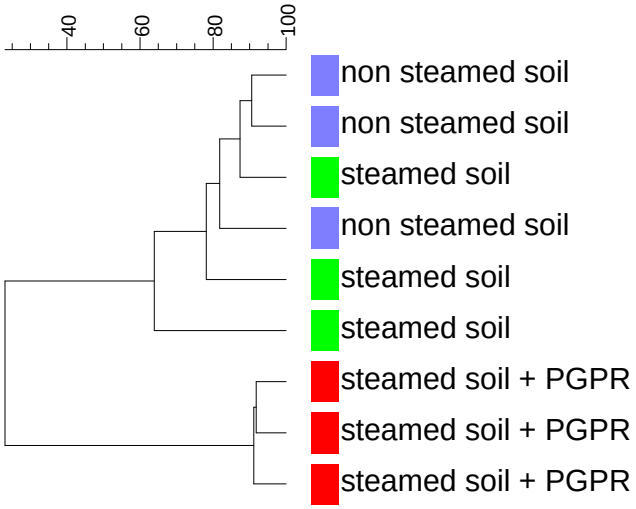


Figure 1. DGGE profile of total rhizpsphere bacterial community of chrysanthemum generated by amplification of 16SrDNA fragments with primers F968GC and 1401R. Three replicates per treatment. The arrows indicated reference marker. The reference marker was composed of 16s rDNA fragements generated from (in order to migration distance): *Pectobacterium carotovorum, Rhizobium radiobacter, Variovorax soli, Pseudomonas fluorescens, Bacillus amyloliquefaciens, Methylobacterium mesophilicum, and Nocardiopsis dassonvillei.*. **a)** Gel picture corresponds to the the first cycle and sampling at five weeks, abc represent the non-steamd soil, def soil steamed once and ghi soil steamed once + PGPR. **b)** The scale represents % of similarity calculated by the Pearson correlation. The dendrogram was constructed using the unweighted pair-group method with arithmetic mean (UPGMA). Colors are representative of treatments.

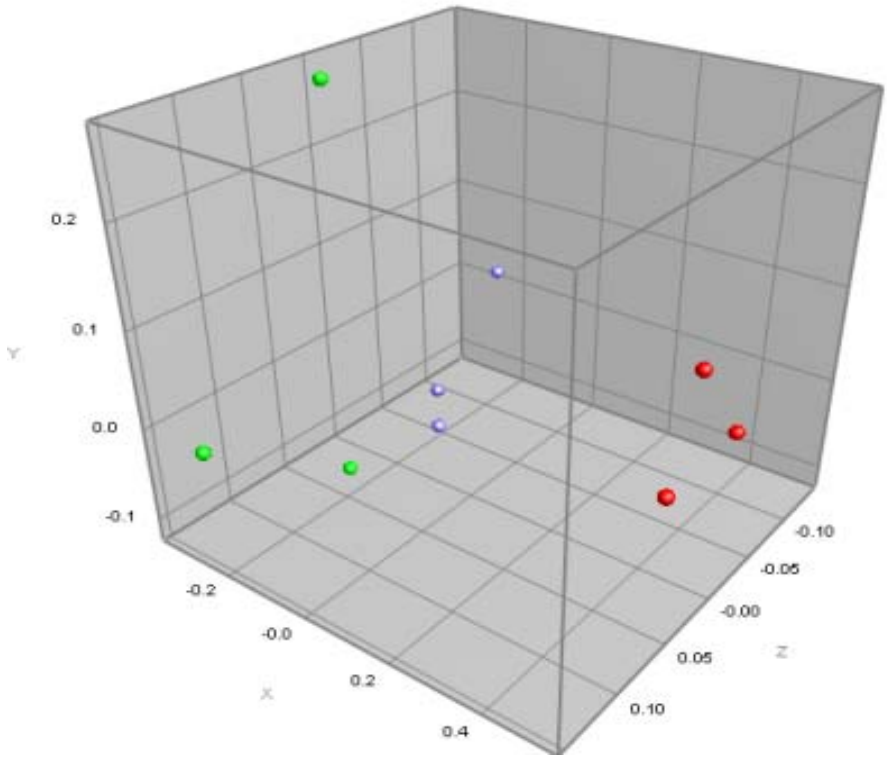
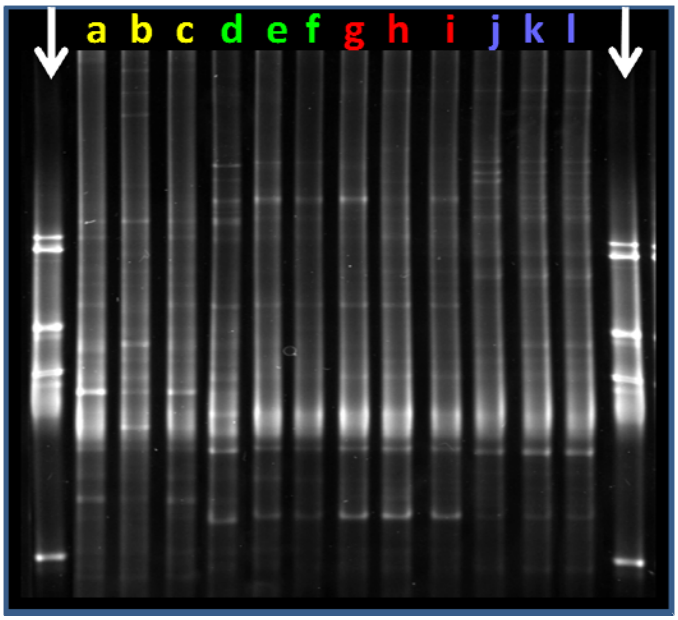


Figure 2. Multi-dimensional Scaling of bacterial community of rhizosphere of chrysanthemum planted in non-steamed soil (purple), steamed soil (green), and steamed soil + PGPR (red).

a)



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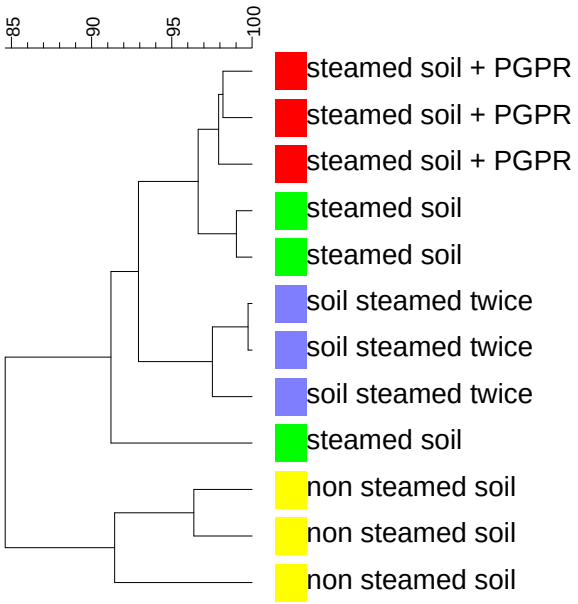


Figure 3. DGGE profile of total rhizpsphere bacterial community of chrysanthemum generated by amplification of 16S rDNA fragments with primers F968GC and 1401R. Three replicates per treatment.The arrows indicated reference marker. **a)** Gel picture corresponds to the the second cycle and sampling at ten weeks, abc represent the non-steamd soil, def soil steamed once, ghi soil steamed once + PGPR, and jkl soil steamed twice. **b)** The scale represents % of similarity calculated by the Pearson correlation. The dendrogram was constructed using the unweighted pair-group method with arithmetic mean (UPGMA). Colors are representative of treatments.

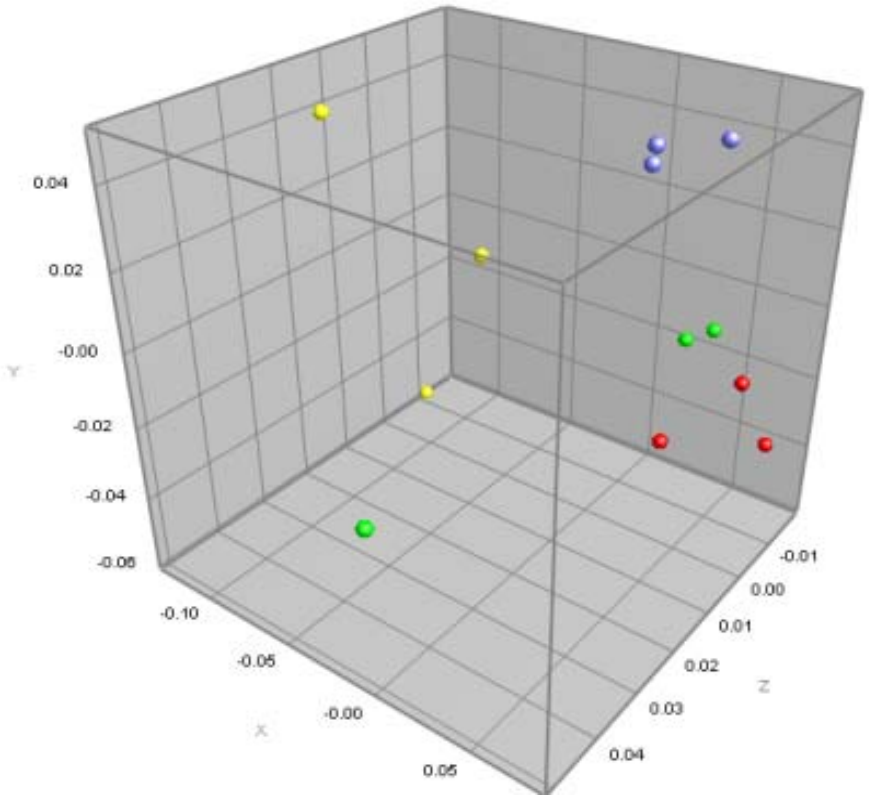
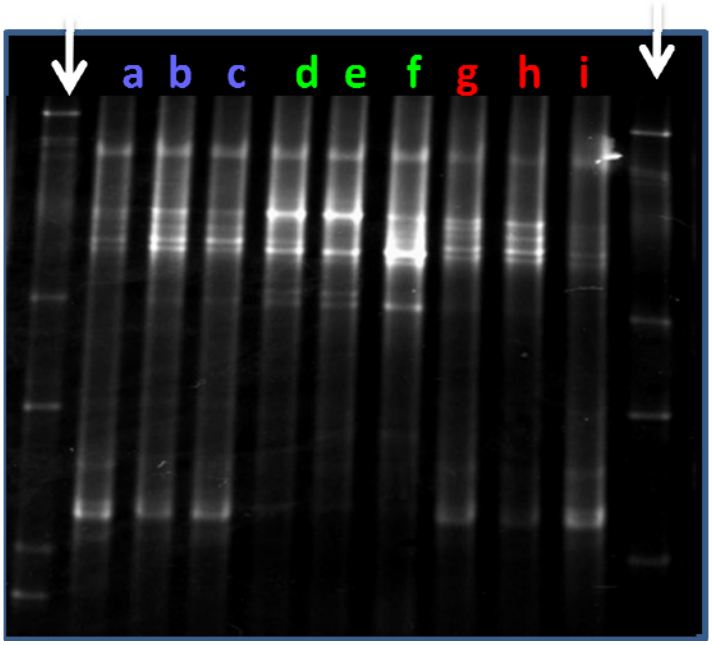


Figure 4. Multi-dimensional Scaling of bacterial community of rhizosphere of chrysanthemum planted in non-steamed soil (yellow), steamed soil (green), steamed soil + PGPR (red), and soil steamed twice (purple).

a)



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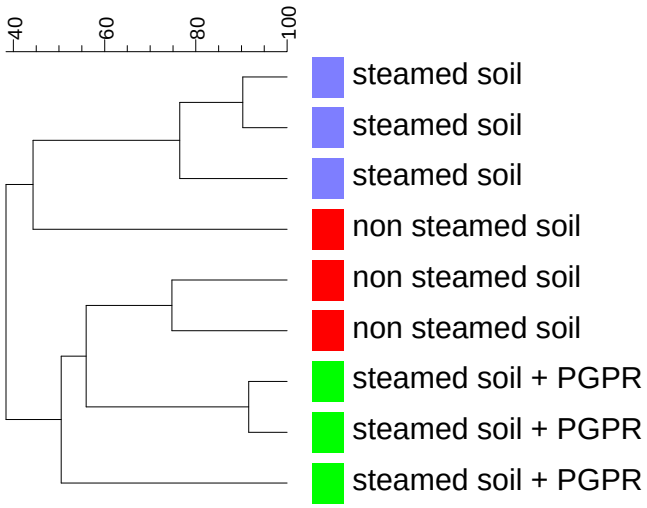


Figure 5. DGGE profile of rhizosphere *Pseudomonas* community of chrysanthemum generated by amplification of 16SrDNA fragments with primers F311Ps and R1459Ps. Three replicates per treatment. The arrows indicated reference marker. **a)** Gel picture corresponds to the the first cycle and sampling at five weeks, abc represent the non-steamd soil, def soil steamed once, and ghi soil steamed once + PGPR at five weeks sampling. **b)** The scale represents % of similarity calculated by the Pearson correlation. The dendrogram was constructed using the unweighted pair-group method with arithmetic mean (UPGMA). Colors are representative of treatments.

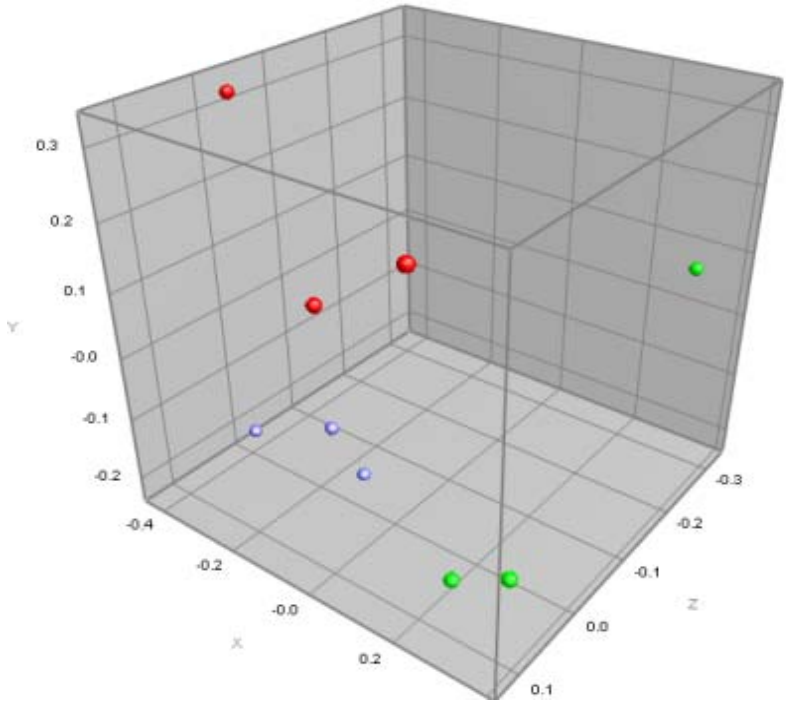
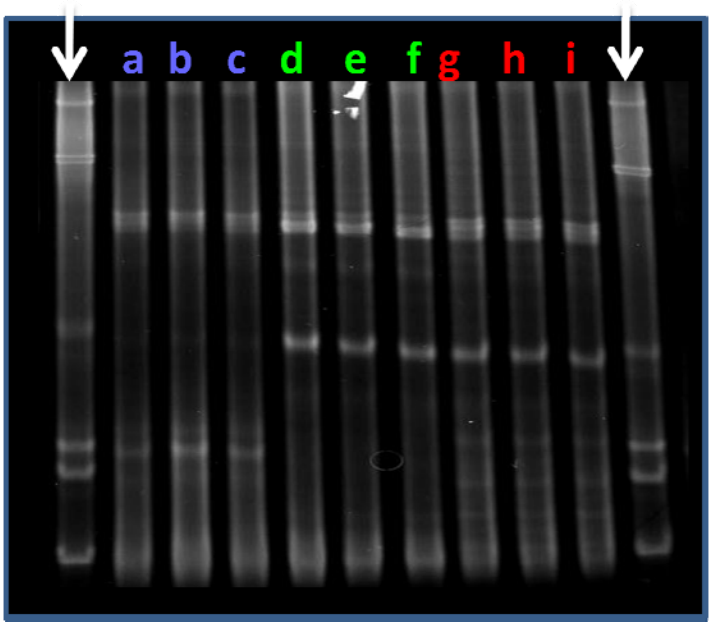


Figure 6. Multi-dimensional Scaling of *Pseudomonas* community of rhizosphere of chrysanthemum planted in non-steamed soil (red), steamed soil (purple), and steamed soil + PGPR (green).

a)



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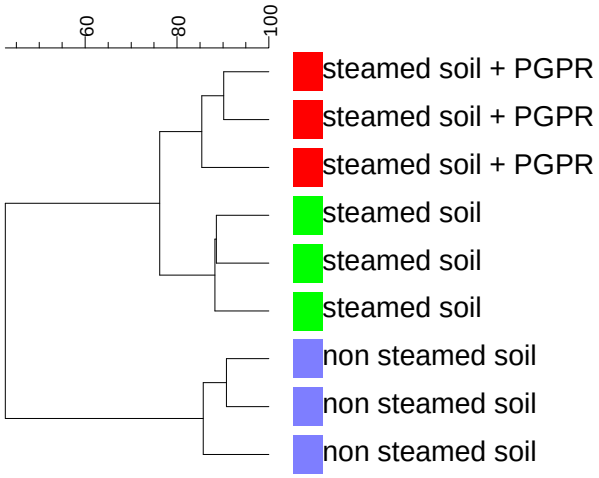


Figure 7. DGGE profile of rhizosphere *Bacillus* and related taxa community of chrysanthemum generated by amplification of 16SrDNA fragments with primers BacF and 1401R. Three replicates per treatment. The arrows indicated reference marker. **a)** Gel picture corresponds to the the first cycle and sampling at five weeks, abc represent the non-steamd soil, def soil steamed once, and ghi soil steamed once + PGPR. **b)** The scale represents % of similarity calculated by the Pearson correlation. The dendrogram was constructed using the unweighted pair-group method with arithmetic mean (UPGMA). Colors are representative of treatments.

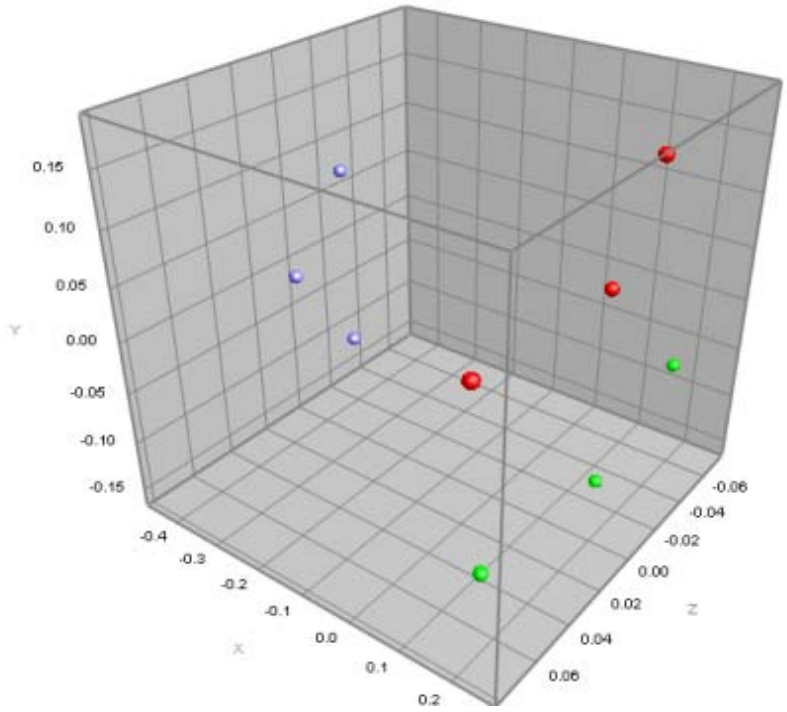
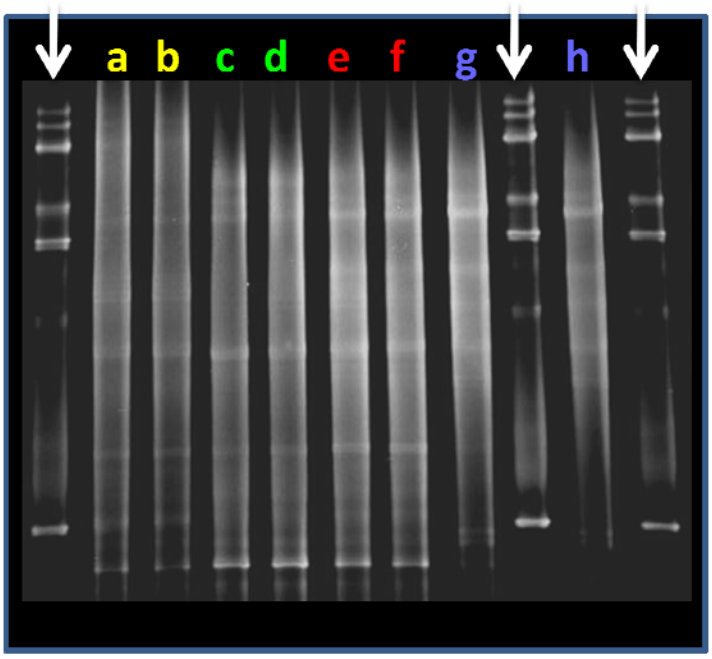


Figure 8. Multi-dimensional Scaling of *Bacillus* and related taxa community of rhizosphere of chrysanthemum planted in non-steamed soil (purple), steamed soil (green), and steamed soil + PGPR (red).

a)



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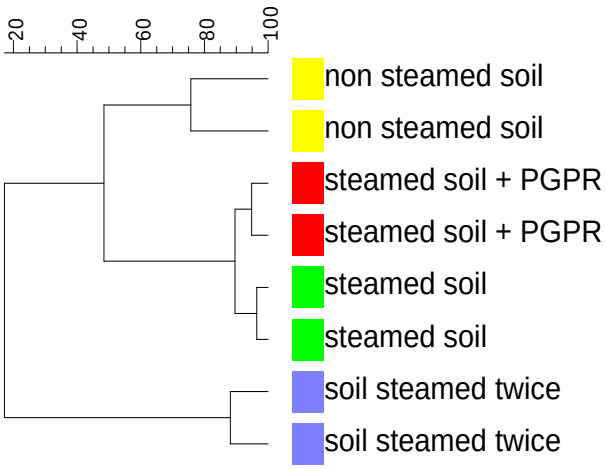

Figure 9. DGGE profile of rhizosphere *Bacillus* and related taxa community of chrysanthemum generated by amplification of 16SrDNA fragments with primers BacF and 1401R. Two replicates per treatment. The arrows indicated reference marker. **a)** Gel picture corresponds to the the second cycle and sampling at ten weeks, ab represent the non-steamd soil, cd soil steamed once, ef soil steamed once + PGPR, and gh soil steamed twice**. b)** The scale represents % of similarity calculated by the Pearson correlation. The dendrogram was constructed using the unweighted pair-group method with arithmetic mean (UPGMA). Colors are representative of treatments.

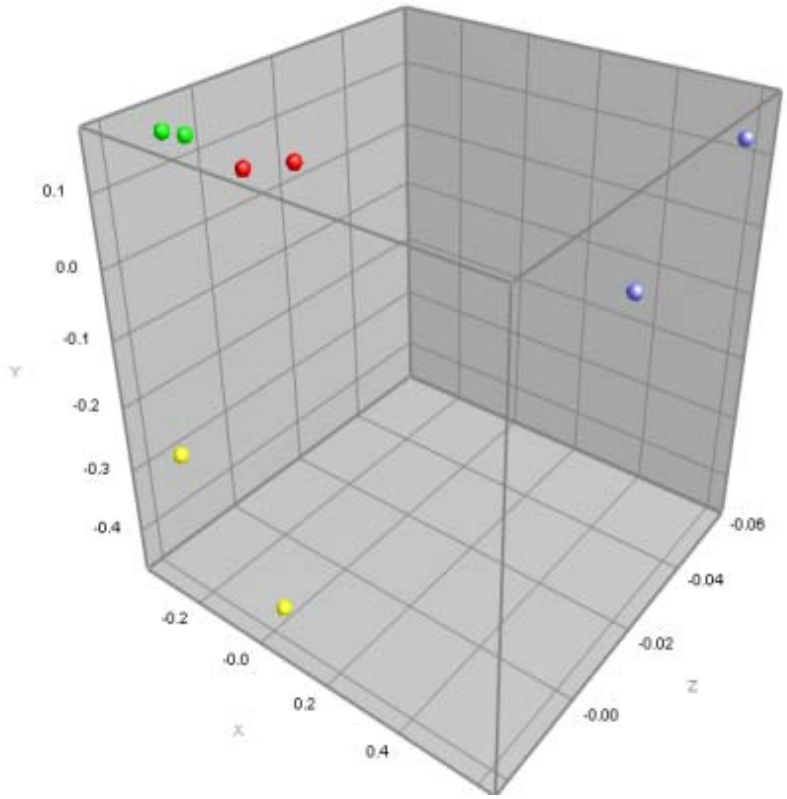

Figure 10. Multi-dimensional Scaling of *Bacillus* and related taxa community of rhizosphere of chrysanthemum planted in non-steamed soil (yellow), steamed soil (green), steamed soil + PGPR (red), and soil steamed twice (purple).

Appendix 1. Chapter II. Greenhouse assay. *P*-value for contrasts of treatment means of bacterial population in the rhizosphere and endophytes of chrysanthemum, five weeks after transplant for cycle 1 and ten weeks after transplant for cycle 2.

	Rhizosphere (cfu, g^{-1}dry soil)						Endophytes (cfu, g^{-1}root)					
	log_e(Total bacteria cfu)		log_e(Pseudomonads cfu)		log_e(AEFB cfu)		log_e(Total bacteria cfu)		log_e(Pseudomonads cfu)		log_e(AEFB cfu)	
	Cycle 1	Cycle 2	Cycle 1	Cycle 2	Cycle 1	Cycle 2	Cycle 1	Cycle 2	Cycle 1		Cycle 1	Cycle 2
Contrasts												
1 vs. 2	0.007		0.11		0.099		0.86		0.18		0.31	
1 vs. 3	0.012		0.03		0.965		0.16		0.26		0.001	
2 vs. 3	0.832	0.35	0.17	1	0.109	0	0.08	0.87	0.05		0.006	0
1 vs. 4		0.63		0		0.197		0.06				0.01
2 vs. 4		0		0		0.081		0.02				0.22

1= non-steamed control, 2= soil steamed once, 3=soil steamed once + PGPR, 4=soil steamed twice

Appendix 2. *Blast nearest neighbor and accession number of fluorescent pseudomonads isolated* from the rhizosphere of chrysanthemum

Treatment	Cycle	Sampling week	Blast nearest neighbor	Number of isolates	Accession no. [a]
Control	1	5	*Pseudomonas fluorescens*	5	GU726880
Control	1	5	*Pseudomonas fluorescens*	5	GU177878[E]
Control	1	5	*Pseudomonas monteilii*	2	EF600861
Control	1	5	*Pseudomonas putida*	12	GU396284
Control	1	5	*Pseudomonas putida*	4	GU396283
Control	1	5	*Pseudomonas putida*	3	GQ200822 [E]
Control	1	5	*Pseudomonas putida*	2	GU329915
Control	1	5	*Pseudomonas putida*	1	EU475959
Control	1	5	*Pseudomonas putida*	2	GU335250[E]
Control	1	5	*Pseudomonas putida*	2	GU726875
Control	1	5	*Pseudomonas putida*	2	GU377179
steam once	1	5	*Pseudomonas fluorescens*	8	CP000094[E]
steam once	1	5	*Pseudomonas fluorescens*	8	GU726880
steam once	1	5	*Pseudomonas fluorescens*	1	EU169164
steam once	1	5	*Pseudomonas putida*	3	GU339288
steam once	1	5	*Pseudomonas putida*	3	GU372963
steam once	1	5	*Pseudomonas putida*	1	AB512773
steam once	1	5	*Pseudomonas putida*	13	GU396284 [E]
steam once	1	5	*Pseudomonas putida*	3	GQ200822
steam once + PGPR	1	5	*Pseudomonas fluorescens*	4	GU726880
steam once + PGPR	1	5	*Pseudomonas fluorescens*	3	CP000094 [E]
steam once + PGPR	1	5	*Pseudomonas fluorescens*	1	GU367870

steam once + PGPR	1	5	*Pseudomonas fulva*	1	AM411071
steam once + PGPR	1	5	*Pseudomonas fulva*	1	EF600885
steam once + PGPR	1	5	*Pseudomonas jessenii*	1	FM209481
steam once + PGPR	1	5	*Pseudomonas monteilii*	5	EF600861
steam once + PGPR	1	5	*Pseudomonas putida*	8	GU396284
steam once + PGPR	1	5	*Pseudomonas putida*	1	GU377179
steam once + PGPR	1	5	*Pseudomonas putida*	1	GU372963
steam once + PGPR	1	5	*Pseudomonas putida*	1	GQ200822
steam once + PGPR	1	5	*Pseudomonas putida*	1	EU826028
steam once + PGPR	1	5	*Pseudomonas putida*	1	GU335250
steam once + PGPR	1	5	*Pseudomonas putida*	5	GU248219
steam once + PGPR	1	5	*Pseudomonas putida*	1	DQ112332
steam once + PGPR	1	5	*Pseudomonas putida*	1	GU726875
steam once + PGPR	1	5	*Pseudomonas putida*	2	GU817324
steam once + PGPR	1	5	*Pseudomonas putida*	1	AY918068
steam once + PGPR	1	5	*Pseudomonas putida*	1	FJ976589
Control	1	10	*Pseudomonas fluorescens*	1	FJ608707
Control	1	10	*Pseudomonas fulva*	1	EF600885
Control	1	10	*Pseudomonas mosselii*	1	EU244714
Control	1	10	*Pseudomonas mosselii*	1	EF178445
Control	1	10	*Pseudomonas putida*	5	EU826028
Control	1	10	*Pseudomonas putida*	3	GU335250
Control	1	10	*Pseudomonas putida*	1	GU396283[E]
Control	1	10	*Pseudomonas putida*	1	GU396284
steam once	1	10	*Pseudomonas fulva*	1	EF600885

steam once	1	10	*Pseudomonas monteilii*	7	EF600861
steam once	1	10	*Pseudomonas putida*	3	GU339288
steam once	1	10	*Pseudomonas putida*	1	GQ200822
steam once	1	10	*Pseudomonas putida*	1	GU726875
steam once	1	10	*Pseudomonas putida*	1	GU828030
steam once + PGPR	1	10	*Pseudomonas citronellolis*	2	FJ462715
steam once + PGPR	1	10	*Pseudomonas monteilii*	2	EF600861
steam once + PGPR	1	10	*Pseudomonas putida*	3	GU339288
steam once + PGPR	1	10	*Pseudomonas putida*	5	GU396284
steam once + PGPR	1	10	*Pseudomonas putida*	1	GU726875
steam once + PGPR	1	10	*Pseudomonas putida*	1	GU073466
steam twice	2	10	*Pseudomonas citronellolis*	7	AM088480[E]
steam twice	2	10	*Pseudomonas citronellolis*	3	FJ462715[E]
steam twice	2	10	*Pseudomonas citronellolis*	2	AB021396[E]
steam twice	2	10	*Pseudomonas citronellolis*	1	NR_026533
steam twice	2	10	*Pseudomonas citronellolis*	1	FJ544978[E]

[a] GenBank sequence accession number of most closely related bacterial species.

[E] This most related species were also obtained for the blast analysis of the endophytic pseudomonads

Appendix 3. Blast nearest neighbor and accession number of endophytic fluorescent pseudomonads isolated from chrysanthemum

Treatment	Cycle	Sampling week	Blast nearest neighbor	Number of isolates	Accession no.
Control	1	5	*Pseudomonas putida*	4	GU396283
Control	1	5	*Pseudomonas putida*	2	GU335250
Control	1	5	*Pseudomonas putida*	1	GQ200822
Control	1	5	*Pseudomonas fluorescens*	1	CP000094
Control	1	5	*Pseudomonas citronellolis*	1	AY972255
Control	1	5	*Pseudomonas putida*	7	DQ133506
Steam once	1	5	*Pseudomonas fluorescens*	16	CP000094
Steam once	1	5	*Pseudomonas putida*	5	GU396284
Steam once	1	5	*Pseudomonas fluorescens*	3	GU177878
Steam once	1	5	*Pseudomonas fluorescens*	4	EF690400
Steam once	1	5	*Pseudomonas fluorescens*	3	EF690400
Steam once	1	5	*Pseudomonas putida*	5	GU396284
Steam once	1	5	*Pseudomonas putida*	4	DQ232745
Steam once	1	5	*Pseudomonas putida*	1	DQ232738
Steam once + PGPR	1	5	*Pseudomonas fluorescens*	1	DQ922745
Steam once + PGPR	1	5	*Pseudomonas fluorescens*	1	CP000094
Steam once + PGPR	1	5	*Pseudomonas putida*	7	AY973267
Control	1	10	*Pseudomonas citronellolis*	1	FJ544978
Steam once	1	10	*Pseudomonas citronellolis*	2	AM088480
Steam once	1	10	*Pseudomonas citronellolis*	2	AB021396
Steam once	1	10	*Pseudomonas citronellolis*	1	FJ544978
Steam once + PGPR	1	10	*Pseudomonas putida*	3	GU396284
Steam once + PGPR	1	10	*Pseudomonas putida*	2	GQ200822
Steam once + PGPR	1	10	*Pseudomonas citronellolis*	1	FJ462715

Appendix 4. Level of IAA production, HCN, HR and PA of fluorescent pseudomonads isolated from the rhizosphere of chrysanthemum.

Blast	IAA ordinal scale	IAA (µg/ml/OD 600 nm)	HCN	HR	PA
Pseudomonas fluorescens	low	25.37630547	1	0	0
Pseudomonas fluorescens	low	22.39296397	1	0	1
Pseudomonas monteilii	low	26.1859	0	0	0
Pseudomonas putida	low	36.99015471	0	0	0
Pseudomonas putida	low	49.30633357	0	0	0
Pseudomonas putida	low	17.38638887	0	0	0
Pseudomonas putida	low	33.61444674	0	0	0
Pseudomonas putida	high	89.15707595	0	0	0
Pseudomonas putida	high	61.18583899	0	0	0
Pseudomonas putida	low	28.76921592	0	0	0
Pseudomonas putida	low	28.08020232	1	0	1
Pseudomonas fluorescens	low	30.01880941	1	0	0
Pseudomonas fluorescens	low	39.69716392	1	0	0
Pseudomonas fluorescens	low	24.70295108	0	0	0
Pseudomonas putida	low	51.19230277	0	0	1
Pseudomonas putida	low	45.58539375	0	0	1
Pseudomonas putida	low	26.287022	0	0	1
Pseudomonas putida	low	27.95751665	0	0	1
Pseudomonas putida	low	34.86702733	0	0	1
Pseudomonas fluorescens	low	38.33411471	1	0	1
Pseudomonas fluorescens	low	36.48076489	1	0	1
Pseudomonas fluorescens	low	54.38882496	1	0	1
Pseudomonas fulva	low	44.35864248	1	0	1
Pseudomonas fulva	low	41.14721972	1	0	1

Pseudomonas jessenii	low	42.09563994	0	0	1
Pseudomonas monteilii	high	72.3524936	0	0	1
Pseudomonas putida	high	71.12526539	0	0	1
Pseudomonas putida	low	25.98436828	1	0	1
Pseudomonas putida	low	33.27539692	0	0	1
Pseudomonas putida	high	62.5415835	0	0	1
Pseudomonas putida	low	44.59824545	0	0	1
Pseudomonas putida	high	68.90718283	0	0	1
Pseudomonas putida	low	57.46812697	1	0	1
Pseudomonas putida	low	34.13276831	0	0	1
Pseudomonas putida	low	50.16472254	0	0	1
Pseudomonas putida	high	74.01107595	0	0	1
Pseudomonas putida	low	40.92758172	0	0	1
Pseudomonas putida	low	35.20938586	1	0	1
Pseudomonas fluorescens	low	32.45545624	1	0	1
Pseudomonas fulva	low	53.60169244	0	0	1
Pseudomonas mosselii	low	37.20140535	1	0	1
Pseudomonas mosselii	low	47.11349628	1	0	1
Pseudomonas putida	high	72.33552118	0	0	1
Pseudomonas putida	high	81.86174923	0	0	1
Pseudomonas putida	high	108.8440253	0	0	1
Pseudomonas putida	high	106.9913427	0	0	1
Pseudomonas fulva	high	99.8915009	0	0	0
Pseudomonas monteilii	low	27.12119313	0	0	1
Pseudomonas putida	high	93.5796556	0	0	0
Pseudomonas putida	high	86.07133799	0	0	1
Pseudomonas putida	high	121.6048915	0	0	1
Pseudomonas putida	high	141.6987709	0	0	1

Pseudomonas citronellolis	low	52.41923065	0	0	0
Pseudomonas monteilii	high	119.374121	0	0	1
Pseudomonas putida	high	117.5983166	0	0	1
Pseudomonas putida	high	88.23006884	0	0	1
Pseudomonas putida	high	127.9159337	0	0	1
Pseudomonas putida	high	79.00725359	0	0	0
Pseudomonas citronellolis	high	87.21124366	0	0	0
Pseudomonas citronellolis	low	58.570414	0	0	1
Pseudomonas citronellolis	high	62.59500774	0	0	0
Pseudomonas citronellolis	high	80.93304673	0	0	1
Pseudomonas citronellolis	high	106.9720715	0	0	0

Appendix 5. Level of IAA production, HCN, HR and PA of endopgytic fluorescent pseudomonads isolated from chrysanthemum.

Blast	IAA ordinal scale	IAA µg/ml/OD 600 nm	HCN	HR	PA
Pseudomonas putida	high	68.53271528	1	0	1
Pseudomonas putida	high	62.1088369	1	0	1
Pseudomonas putida	high	75.92497744	1	0	1
Pseudomonas fluorescens	high	103.3625845	1	0	1
Pseudomonas citronellolis	high	167.8988835	0	0	0
Pseudomonas putida	high	84.68629609	0	0	1
Pseudomonas fluorescens	low	59.1943872	1	0	0
Pseudomonas putida	high	118.2524886	1	0	0
Pseudomonas fluorescens	high	148.8463955	1	0	1
Pseudomonas fluorescens	high	76.65666691	1	0	1
Pseudomonas fluorescens	low	57.56479807	1	0	1
Pseudomonas putida	high	78.60783883	1	0	0
Pseudomonas putida	low	39.02052003	1	0	1
Pseudomonas putida	low	37.31935295	1	0	1
Pseudomonas fluorescens	high	79.50255385	1	0	1
Pseudomonas fluorescens	high	64.33169817	1	0	0
Pseudomonas putida	low	32.07541576	0	0	1
Pseudomonas citronellolis	high	61.87763713	1	0	1
Pseudomonas citronellolis	high	78.07680034	0	0	1
Pseudomonas citronellolis	high	91.17280399	0	0	0
Pseudomonas citronellolis	high	86.0265733	0	0	0
Pseudomonas putida	high	296.9713036	0	0	1
Pseudomonas putida	high	105.564346	0	0	1
Pseudomonas citronellolis	high	89.78995688	0	0	1

Appendix 6. Partial sequence of the 16S ribosomal RNA for *Pseudomonas* isolated from the rhizosphere and roots of chrysanthemum

Pseudomonas putida strain 63C9- ACCESSION EU475959

808bp

GCAGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCT

GGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCG

GGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCC

GTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG

GGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG

CACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGC

TAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCG

TAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGA

GTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAA

GGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG

TCCACGCCGTAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTA

Pseudomonas putida strain: S5- ACCESSION AB512773

946bp

TCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTA

GTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCC

TTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAA

CTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA

ATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACT

TTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAAC

TCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGG

TGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTAC

GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCG

ACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA

CGCCGTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCG

CCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT

TTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAG

Pseudomonas fluorescens strain 1408-ACCESSION GU726880

1390bp

TCGAGCGGATGAAGGGAGCTTGCTCCTGAATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTA

GTGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCT

TGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAAC

TGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA

TATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTT

TAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGGCTAACT

CTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGT

GGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTATG

GTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCNA

CCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC

GCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGC

CTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT

TAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCCAGAGATGGATTGGTGCCTT

CGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACG

AGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAG

GAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAA

AGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGAC

TGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACAC

CGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCCTTCGGGA

Pseudomonas fluorescens Pf0-1-ACCESSION CP000094

1397bp

TGCAGTCGAGCGGATGAAAGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCC

TGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTC

GGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATC

CGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT

GGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAA

GCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGG

CTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGC

GTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAG

AGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGA

AGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA

GTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTT

GACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT

GTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCCAGAGATGGATTGG

TGCCTTCGGGAGCATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC

GTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAA

CCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCG

GTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAA

CTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGT

ACACACCGCCCGTCACANCCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCCTTCGGGA

Pseudomonas fluorescens strain BIT-ACCESSION GU367870

934bp

CACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA

GCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACA

AGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAG

TGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC

CTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCAT

TAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTG

GAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCCAGAGATG

GATTGGTGCCTTCGGGAGCATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA

AGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGT

GACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAA

TGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGT

CTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGG

CCTTGTACACTACCGCCCGTCACCACCATGGGAGTGGGTTGCACCA

Pseudomonas fulva strain Z67zhy-ACCESSION AM411071

1274bp

CGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGA

GCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATG

ATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGC

GAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAG

GGTTGTAGATTAATACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCG

CGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGG

ATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTATGGTAGAGGGTGGTGGA

ATTTTCCTGTGTAGCGGTGAAAATGCGTAGATATAGGAAGGGAACACCAGTGGCGAAGGCGACCACCTGGACTG

ATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT

GTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACG

GCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA

ACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGA

CACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTT

GTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT

GACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAG

CCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCG

GAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACTACCGCCCGTCACC

ACCATGGGAGTGGGTT

Pseudomonas jessenii strain T2P28-ACCESSION FM209481

1343bp

TTCCTTGATTCAGCGGCGGNACGGGATGAGTAATGCCTAAGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAA

AGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCT

AGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCA

GTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAA

GCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCA

GTAAGTTAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGT

AATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGT

GAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTACGGTAGAGGGTGGTGGAATTT

CCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGA

CACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACT

AGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAA

GGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA

GAACCTTACCAGGCCTTGACATCCAATGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACATTGAGACAGGT

GCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTA

GTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA

AGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAG

GTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGC

TAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACTCCGCCCGTCACCACCATGGG

AGTGGGTTGCA

Pseudomonas putida strain LW 16S-ACCESSION GU377179

952bp

GTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGT

AGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGC

CTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATCCGTA

ACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG

AATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCAC

TTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAA

CTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAG

GTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTA

TGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGC

GACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC

ACGCCGTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACC

GCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG

TTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATG

Pseudomonas putida strain BA-2 D-2-ACCESSION GU372963

884bp

TCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTA

GTGGGGGACAACGTTCCGAAAGGAGCGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACC

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Pseudomonas putida strain CDd-9-ACCESSION GU248219

1387bp

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Pseudomonas putida strain JSK3-ACCESSION GU817324

1016bp

TGCTCCTTGATTCAGCGGCGGACGGGTGAGCTAATGCCTANAGAATCTGCCTGGTAGTGGGGGACAACGTTTCG
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Pseudomonas putida strain PC30-ACCESSION AY918068

1355bp

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CATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTG

GAGCTAATCCCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAG

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GGTTGCACCAGAAGTAGCTAGTC

Pseudomonas putida strain LCR80-ACCESSION FJ976589

952bp

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TTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAA

CTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA

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CGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCG

CCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT

TTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCCAGAGATGG

Pseudomonas mosselii isolate 52-ACCESSION EU244714

953bp

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Pseudomonas mosselii strain 2R12-ACCESSION EF178445

825bp

GGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGG

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GGAGTACGGCC

Pseudomonas fluorescens strain SB336-ACCESSION FJ608707

811bp

GGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGG

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TATCAGATGAGCCTAGGTCGGATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATCCGTAACTGGTC

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Pseudomonas putida strain SRI156-ACCESSION EU826028

972bp

CAGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTA

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CGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCCGTAACTGGTC

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CAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGG

CCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAA

Pseudomonas putida strain X16-ACCESSION GU335250

948bp

GCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGG

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Pseudomonas putida strain BASUP87-ACCESSION GU396283

991bp

GAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGT

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CTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT

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Pseudomonas fulva strain BFPB87-ACCESSION EF600885

722bp

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CCTGGACTGATACTGACCCTGAGGTGCGAAAGCGTGGGGAGCAAACACGATTAGAT

Pseudomonas putida strain SP2 16S-ACCESSION GQ200822

1398bp

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Pseudomonas monteilii strain BFPB63-ACCESSION EF600861

623bp

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Pseudomonas putida strain EH63-ACCESSION GU339288

929bp

GTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGT

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ACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG

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Pseudomonas putida strain 1389 -ACESSION GU726875

887 bp

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Pseudomonas putida strain TP0701_ACCESSION GU073466

817bp

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CAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTA

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GGAGGAAGGGCAGTAAGNTAATACCTTGCTGTTTTGACGTTTCCGACAGAATAAGCACCGGCTAACTCTGTGCC

AGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGT

TAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTACGGTAGAGG
GTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTG
GACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGA
GTA

Pseudomonas putida strain JGP46- ACCESSION GU396284

926bp

TCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTA
GTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCC
TTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAA
CTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACT
TTAAGTTGGGAGGAAGGGCAGTAAGNTAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAAC
TCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGG
TGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTAC
GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCG
ACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCG
CCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGGCCGCACAAGCGGTGGAGCATGTGGT
TTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTG

Pseudomonas citronellolis strain DSM 50332 - ACCESSION NR_026533

950 bp

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AGTGGGGGACAACGTTCCGAAAGGAGCGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGAC
CTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCGACGATCCGTA
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CTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAG

GTGGTTTGGTAAGATGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCATAACTGCCTGACTAGAGTA

CGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGACAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGG

CGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC

CACGCCGTAAACGATGTCGAGCTAGCCGTTGGGATACCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCG

ACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG

TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCCGGAATTCTGCAG

Pseudomonas citronellolis strain BG 6903-ACCESSION AM088480

1135 bp

CAAGTCGAGCGGATGAAGGGAGCTTGCTCTCTGATTCAGCGGCGGACGGGTGAGTTATTGCCTAGGAATCTGCC

TGGTAGTGGGGGACAACGTTCCGAAAGGAGCGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTC

GGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCGACGATC

CGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT

GGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAA

GCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGG

CTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGC

GTAGGTGGTTTGGTAAGATGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCATAACTGCCTGACTAG

AGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC

CTTACCTGGCCTTGACATGTCCGGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGAATCGGAACACAGGTGCTG

CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTA

CCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTC

ATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGG

AGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGT

AATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGG

GTTGCTCCAGAAGTAGCTAGTCTAA

Pseudomonas citronellolis strain YS-8 16S -ACCESSION FJ462715

876 bp

TCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTCCGAAAGGAGCGCTAA

TACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTA

GCTAGTTGGTGGGGTAAAGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGA

ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAG

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TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGGTAAGATGGATGTGAAATCCCCGG

GCTCAACCTGGGAACTGCATCCATAACTGCCTGACTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCG

GTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACATGAGGTGCG

AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGAT

CCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA

AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA

Pseudomonas citronellolis - ACCESSION AB021396

810 bp

TCGAGCGGATGAAGGGAGCTTGCTCTCTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTA

GTGGGGGACAACGTTCCGAAAGGAGCGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACC

TCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCGACGATCCGTAA

CTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA

ATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACT

TTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAAC

TTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGG

TGGTTTGGTAAGATGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCATAACTGCCTGACTAGAGTAC

GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCG

ACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTNGTCCA

CGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCANNTAACGCGATAAGTCG

Pseudomonas citronellolis strain DFS35-4 -ACCESSION FJ544978

919 bp

CTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGG

CCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCGCGCAGTCGAGC

GGATGAAGGGAGCTTGCTCCCGGATTCAGCGGCGGACGGGTGAGTTAATGCCTAGGAATCTGCCTGGTAGTGGG

GGACAACGTTCCGAAAGGAGCGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACG

CTATCAGATGAGCCTAGGTCGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCGTAACTGGT

CTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT

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AGGGTGGTGGAATTTCCTTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACC

TGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT

AAACGATGTCGACTAGCCGTTGGGATCCTTG

Endophyte fluorescent pseudomonas

Pseudomonas putida strain GM6-ACCESSION DQ133506

964bp

AGTCGAGCGGATGAAGAGAGCTTGCTCTCTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGG

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AAGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTTTGACGTTACCGACAGAATAAGCACC
GGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
GCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACAAGCT
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AT

Pseudomonas citronellolis - ACCESSION FJ544978

807 bp

GCGGATGAAGGGAGCTTGCTCNCTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGG
GGGACAACGTTCCGAAAGGAGCGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCAC
GCTATCAGATGAGCCTAGGTCGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCGTAACTGG
TCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT
TGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAA
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TTGGTAAGATGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCATAACTGCCTGACTAGAGTACGGTA
GAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCA
CCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATANCCCTGGTAGTCCACCG
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Pseudomonas citronellolis strain R13 - ACCESSION AY972255

676 bp

CGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCGACGATCCGTAACTG
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TTGNACAATGGGCGNAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTA
AGTTGGGAGGAAGGCCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTC

GTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGG

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TCGNCCGCCT

Pseudomonas fluorescens -ACCESSION EF690400

807bp

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CGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGAT

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CGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTA

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AAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT

AGTCCAGCGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCAT

Pseudomonas putida strain JH17-ACCESSION DQ232745

822p

GCAGTCGAGCGGATGACGGGAGCTTGCTCCCTGAATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCC

TGGTAGTGGGGGACAACGTCTCGAAAGGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTC

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GCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGG

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GTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACAAGCTAG

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GTCCACGCCGTAAACGATGTCAACTAGCCCGTTGGGAGCCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAG

TGCCGCCT

Pseudomonas putida strain JH10-ACCESSION DQ232738

955bp

CATACAATGCAGTCGAGCGGATGACGGGAGCTTGCTCCCTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGA

ATCTGCCTGGTAGTGGGGGACAACGTCTCGAAAGGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGTC

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Pseudomonas fluorescens -ACCESSION DQ922745

789bp

GGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGT

TTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGAT

GAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGA

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CGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTT

GGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTATGGTAGAGGGTGGTG

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TACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG

TCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCAT

Pseudomonas putida -ACCESSION AY973267

809bp

TGCAGTCGAGCGGATGAAGAGAGCTTGCTCTCTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCC

TGGTAGTGGGGGACAACGTCTCGAAAGGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTC

GGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATC

CGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT

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GCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGG

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GTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACCGCAT

Appendix 7

AUBURN UNIVERSITY

SOIL TESTING LABORATORY

SOIL ANALYSIS REPORT

Marleny Cadena
Plant Pathology
209 Life Science Building
Auburn University

ALFA Agricultural Service & Research Building
961 S. Donahue Dr.
Auburn University, Auburn, AL 36849-5411
Phone (334)844-3958
Soillab@auburn.edu

SPECIAL LAB I.D. : 09.S1159 -S1160				DATE: 6-19-09		
UNITS: ppm & % in soil						

	ppm	ppm	ppm	ppm	ppm	ppm
Sample I.D.	Ca	K	Mg	P	Al	B
Control	954	159	120	14	163	1.0
ST-Soil	1030	179	124	13	145	0.7

	ppm	ppm	ppm	ppm	ppm	
Sample I.D.	Cu	Fe	Mn	Na	Zn	
Control	1.7	71	23	67	3.3	
ST-Soil	1.6	57	57	75	63	

	%	%				
Sample I.D.	N	C			pH	
Control	0.086	4.74			6.22	
ST-Soil	0.047	0.44			6.24	

	%	%	%		cm^3/cm^3	
Sample I.D.	Sand	Silt	Clay	Textural Class	H_2O avail.	
Control	61.8	25.8	12.5	Sandy Loam	0.11	
ST-Soil	61.0	25.9	13.1	Sandy Loam	0.11	