UNIVERSITÀ DEGLI STUDI DI MILANO

PhD School of Chemistry, Biochemistry and Ecology of Pesticide

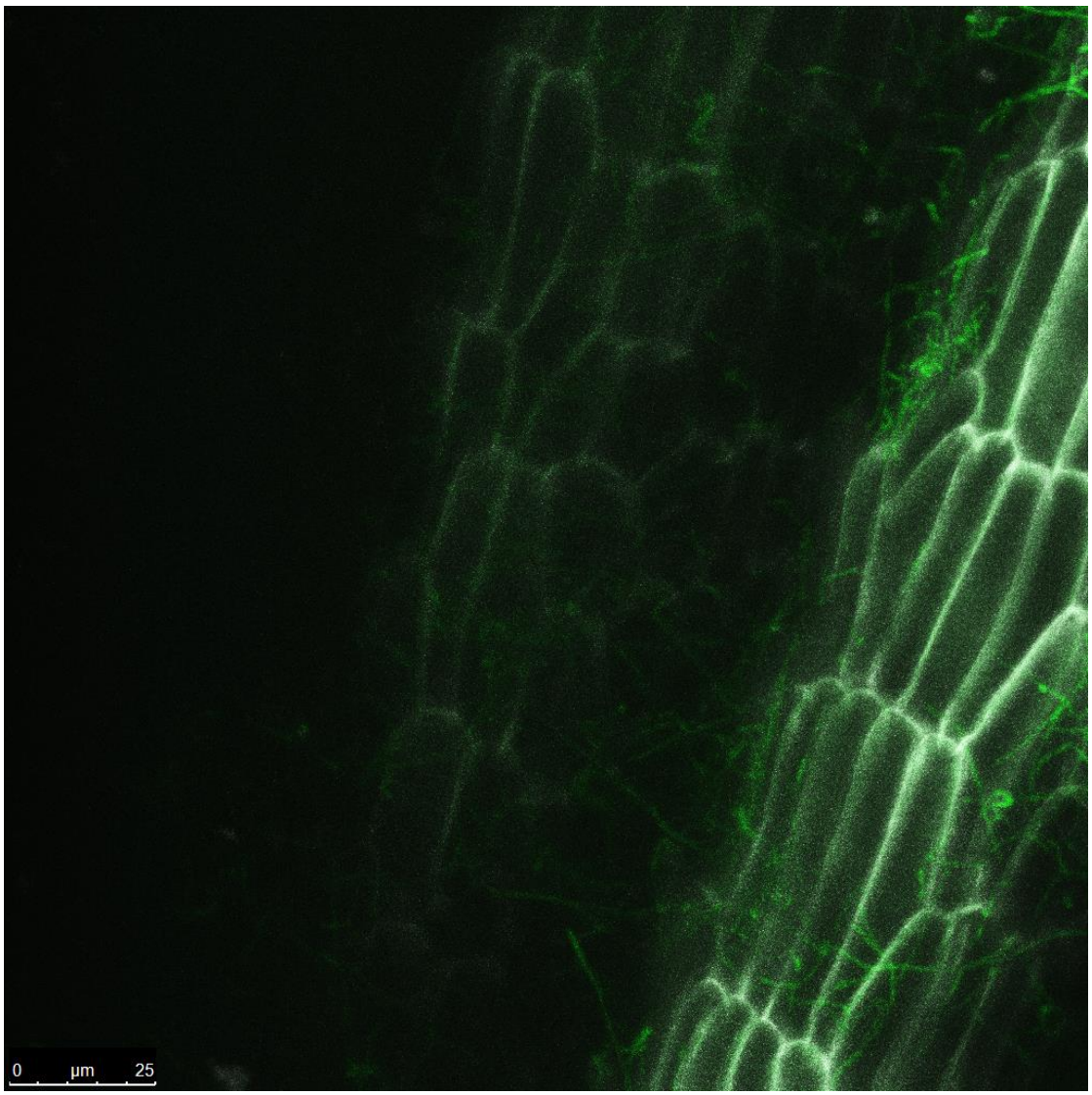
(CBEA)

DOCTORAL DISSERTATION

Tagging biocontrol *Streptomyces* to study lettuce colonization

Xiaoyulong Chen

To be presented with the permission of the examining board, 28th cycle of the CBEA PhD school, for public examination at Aula Magna C03 (Room C03), Faculty of Agricultural and food sciences, University of Milan, on 17.12.2015.



Cover figure: Colonization of lettuce root by *Streptomyces cyaneus* ZEA17I, examined by confocal laser scanning microscopy.

Supervisor:

Professor Paolo Cortesi

Department of Food, Environmental, and Nutritional Sciences

University of Milan, Italy

Co-supervisor:

Professor Marco Saracchi

Department of Food, Environmental, and Nutritional Sciences

University of Milan, Italy

Coordinator:

Professor Daniele Daffonchio

Department of Food, Environmental, and Nutritional Sciences

University of Milan, Italy

Examination board (ordered by first letter of the family name):

1. **Professor Paolo Bertolini**

   Department of Agricultural Sciences

   University of Bologna, Italy

2. **Dr. Jonas Bengtsson**

   Department of Zoology

   Stockholm University, Sweden

3. **Professor Daniele Daffonchio**

   Department of Food, Environmental, and Nutritional Sciences

   University of Milan, Italy

4. **Professor George Tsiamis**

   Dep. of Environmental and Nutritional Resources Management

   University of Patras, Greece

5. **Dr. Laura Polito**

   Institute of Molecular Science and Technology, CNR, Italy

Contents

List of original publications 1
List of conferences attended 2
List of awards 3
Invitation for peer-review 3
Abbreviations 4
List of figures 5
List of tables 6
Abstract 7
Introduction 9
Lettuce 9
Towards sustainable agriculture: the application of biological control agents 14
Streptomyces spp. in nature, and their functions in agriculture 17
The crucial role of plant colonization by microorganisms in biocontrol mechanisms 20
Antibiotic resistance markers as fundamental tools to study microbe colonization dynamics 24
EGFP marker as a convenient approach to visualize colonization patterns of beneficial microbes 25
FISH, a cytogenetic and useful technique to study plant-microbe interactions 27
Aim of the study 29
Materials and methods 31
1. Transformation of the *Streptomyces* strains with the pIJ8641 construct 31
1.1 Conjugation 31
1.2 PCR amplification of the EGFP-transgene 32
1.3 Microscopic observation of EGFP expression 32
2. Impact of the transformation on fitness of *Streptomyces* strains 33
2.1 Antagonistic activity against *Sclerotinia sclerotiorum* 33
2.2 Sporulation 34
2.3 Mycelium biomass growth 34

2.4 Production of secondary metabolites 35
3. Lettuce colonization dynamics by two tagged *Streptomyces* strains 36
3.1 Lettuce bulk soil colonization 37
3.2 Lettuce rhizosphere colonization 37
3.3 Lettuce inner-root colonization 38
4. CLSM observation of lettuce root colonization by *S. cyaneus* EGFP-ZEA17I 38
5. CLSM observation in combination with fluorescence in situ hybridization (FISH) 40
6. Statistical analysis 41
Results 42
1. Transformation of *Streptomyces* strains with the pIJ8641 construct 42
1.1 Conjugation 42
1.2 PCR amplification of the EGFP-transgene 43
1.3 Microscopic observation of EGFP expression 43
2. Impact of the transformation on fitness of *Streptomyces* strains 45
2.1 Antagonistic activity 45
2.2 Sporulation 46
2.3 Mycelium biomass growth 47
2.4 Production of secondary metabolites 48
3. Lettuce colonization dynamics by two tagged *Streptomyces* strains 50
3.1 Bulk soil colonization 50
3.2 Lettuce rhizosphere colonization 51
3.3 Rhizosphere/bulk soil proportion 53
3.4 Lettuce inner-root colonization 53
4. CLSM observation of lettuce colonization by EGFP-ZEA17I 54
4.1 One-week-old plant 54
4.2 Two-week-old plant 56
5. CLSM observation of lettuce colonization by EGFP-ZEA17I in combination with FISH 57
5.1 One-week-old plant 57
5.2 Two-week-old plant 59

Discussion 61
Conclusions: 68
Acknowledgements 69
References 72
Appendix 89
Publication No.1: 89
Publication No.2: 89
Publication No.3: 89
Publication No.4: 89
Award No.1: 89
Award No.2: 89

List of original publications

This dissertation is based on the following publications. The publications have been reprinted with the kind permission of the publishers.

1. Bonaldi M, Chen X*, Kunova A, Pizzatti C, Saracchi M, Cortesi P. 2015. Colonization of lettuce rhizosphere and roots by tagged *Streptomyces*. *Frontiers in Microbiology*. 6:25. *Co-first author

2. Chen X, Bonaldi M, Kunova A, Pizzatti C, Saracchi M, Sardi P, Cortesi P. 2015. Labeling promising biological control streptomycetes with EGFP. *IOBC-WPRS Bulletin*. In press

3. Bonaldi M, Chen X*, Erlacher A, Kunova A, Pizzatti C, Saracchi M, Berg, G, Cortesi P. 2015. Manuscript. *Co-first author

4. Bonaldi M, Saracchi M, Kunova A, Chen X, Pizzatti C, Cortesi P. Selection of *Streptomyces* against soil borne pathogens by a standardized dual culture assay and evaluation of their effects on plant growth. 2015. Submitted

List of conferences attended

This dissertation related work was partially reported or communicated in the following conferences:

1. Milan, Italy, January 2014 - IX Doctorate Workshop in Chemistry, Biochemistry and Ecology of Plant Protection Products and Xenobiotics (Oral Presentation)

2. Milan, Italy, March 2014 - Peking University - Unimi Symposium on Leveraging International Expertise: The Challenge of Food Security

3. Uppsala, Sweden, June 2014 - XIII Conference of the International Organization of Biological Control on Plant Disease (*Best Poster Award*)

4. Uppsala, Sweden, June 2014 - 2nd International Symposium on Biocontrol of Grapevine Diseases

5. PISA, Italy, September 2014 - XX meeting of Italian Phytopathological Society

6. Milan, Italy, December 2014 - X Doctorate Workshop in Chemistry, Biochemistry and Ecology of Plant Protection Products and Xenobiotics (Oral Presentation)

7. Milan, Italy, June 2015 - 13th International Symposium on Bacteria Genetics and Ecology

8. Graz, Austria, July 2015 - 14th Doc-Day Symposium in Molecular Biosciences and Biotechnology

9. Turin, Italy, September 2015 - XXI meeting of Italian Phytopathological Society (representative speaker of "Giovani in Formazione" grants holds)

10. Graz, Austria, October 2015 - International Inter-kingdom Interactions and Biocontrol Workshop (offered talk by organizer)

11. Budapest, Hungary, October 2015 - WG3 meeting: European COST Action FA1103: Endophytes in Biotechnology and Agriculture (invited oral presentation)

12. E-conference, organized by FAO, October 2015 - Utilization of Food Loss and Waste as well as Non-Food Parts as Livestock Feed

List of awards

This dissertation related work contributed to the following awards and high-merits competitions:

1. Poster Award of the XIII Conference of the International Organization of Biological Control on Plant Disease Link: Poster Award

2. Winner of the "GIOVANI IN FORMAZIONE (Young Researcher)" Grant of XXI Meeting of Italian Phytopathological Society Link: Young Researcher Grant

3. Candidate of the Chinese Government Award for Outstanding Self-financed Student aboard in 2015 Link: Candidates

4. Candidate of the Chinese Government Award for Outstanding Self-financed Student aboard in 2014 Link: Candidates

Invitation for peer-review

Due to the relevance of this dissertation work in *Streptomyces* and biocontrol with other researchers' manuscripts, Xiaoyulong Chen was invited by international journals (with impact factors) twice for peer-review work:

1. *Entomological Research* ISSN: 1748-5967

2. *Biological Control* ISSN: 1049-9644

Abbreviations

BCAs	Biological control agents
GFP	Green fluorescent protein
EGFP	Enhanced GFP
FISH	Fluorescence in situ hybridization
CLSM	Confocal laser scanning microscopy
FAO	Food and Agriculture Organization
BCA	Biological control agent
IPM	Integrated pest management
PGP	Plant growth promotion
PGPB	Plant growth promotion bacteria
CFU	Colony-forming units
CZY	Czapek
WA	Water agar
PBS	Phosphate-buffered saline
FA %	Formamide concentration
SEM	Scanning electronic microscopy

List of figures

Figure 1. Disease cycle of *Sclerotinia Sclerotiorum* and *S. minor* on lettuce (Subbarao, 1998). 12
Figure 2. Life cycle of *Streptomyces* (Kieser *et al.*, 2000). 19
Figure 3. Restriction map of pIJ8641. 33
Figure 4. PCR amplification of the EGFP and 16S rDNA fragments 43
Figure 5. Microscopic observation of EGFP expression in transformed *Streptomyces* strains. 44
Figure 6. Mycelium growth of the wild-type and its corresponding transformed (EGFP-) strains. 48
Figure 7. Comparison of population dynamics between two transformed (EGFP-) *Streptomyces* strains in lettuce bulk soil. 51
Figure 8. Comparison of population dynamics of two transformed (EGFP-) *Streptomyces* strains in lettuce rhizosphere. 52
Figure 9. Comparison of population dynamics of two transformed (EGFP-) *Streptomyces* strains in lettuce inner-root tissue. 54
Figure 10. CLSM observations of lettuce root colonization by EGFP-ZEA17I, within one week after bacterization. 55
Figure 11. CLSM observations of lettuce root sample A colonized by EGFP-ZEA17I, two weeks after bacterization 56
Figure 12. CLSM observations of lettuce root sample B colonized by EGFP-ZEA17I, two weeks after bacterization 57
Figure 13. CLSM-FISH observations of lettuce root colonization by EGFP-ZEA17I one week after bacterization at four sites. 58
Figure 14. CLSM-FISH observations of lettuce root sample C colonized by EGFP-ZEA17I two week after bacterization. 59
Figure 15. CLSM-FISH observations of lettuce root sample D colonized by EGFP-ZEA17I two week after bacterization. 60

List of tables

Table 1. Probes used in the FISH experiment of this study. 41
Table 2. Conjugation efficiencies of five *Streptomyces* spp. strains and reference strain *S. coelicolor* A3(2) with the pIJ8641 plasmid 42
Table 3. *In vitro* antagonistic activity of transformed (EGFP-) strains against *Sclerotinia sclerotiorum*, compared to their corresponding wild-type strains. 45
Table 4. Sporulation of transformed (EGFP-) strains, compared to their corresponding wild-type strains. 46
Tabel 5. Mycelium biomass growth of *S. exfoliatus* EGFP-FT05W, compared to its wild-type strain. 47
Table 6. Indole-3-acetic acid synthesis in transformed (EGFP-) strains, compared to their corresponding wild-type strains. 49
Table 7. Phosphate solubilization, chitinase, and siderophore production of the wild-type and transformed (EGFP-) *Streptomyces* spp. strains. 50
Table 8. Population dynamics of two transformed (EGFP-) *Streptomyces* strain in lettuce bulk soil. 51
Table 9. Population dynamics of each transformed (EGFP-) *Streptomyces* strain in lettuce rhizosphere. 52
Table 10. Rhizosphere and bulk soil proportions of two transformed (EGFP-) strains. 53
Table 11. Population dynamics of two transformed (EGFP-) *Streptomyces* strains in lettuce inner-root tissues. 54

Abstract

The ability of the biological control agents (BCAs) to colonize plant tissues is an important feature involved in microbe-assisted plant protection. Plant-microbe interaction research increased especially in the last decade thanks to technological revolution. Molecular methods and the development of advanced microscopic techniques allow researchers to explore gene expression and localization of beneficial microorganisms within plants. The green fluorescent protein (GFP) and its modified version, enhanced GFP (EGFP), more adapt for expression in mammalian cells and GC-rich actinomycetes like *Streptomyces*, have been widely used as markers to study gene expression, as well as plant-microbe interactions. Aside fluorescent protein approaches, fluorescence in situ hybridization (FISH) is another frequently used technique to visualize microbial colonization patterns and community composition by application of specific fluorescent probes. Firstly, we transformed five *Streptomyces* strains, which showed strong inhibition activity against *Sclerotinia sclerotiorum*, with the EGFP construct by the conjugation method. The conjugation efficiencies varied between the strains, but were comparable to the reference strain. The fitness of transformed strains was similar to wild-type; the transformants maintained similar sporulation, mycelium growth rate, and the ability to produce important secondary metabolites and lytic enzymes. Secondly, two transformed strains, *Streptomyces cyaneus* ZEA17I, and *Streptomyces* sp. SW06W, were used to study lettuce colonization dynamics by seed coating method. Their spatio-temporal dynamics were determined in sterile substrate. The strains were consistently recovered from lettuce rhizosphere and inner root tissues up to six weeks. Finally, the colonization pattern of lettuce by *Streptomyces cyaneus* ZEA17I was examined by both EGFP and FISH approaches combined with confocal laser scanning microscopy (CLSM). For FISH-CLSM analysis, universal bacteria and *Streptomyces* genus specific probes were used to label *S. cyaneus* ZEA17I. The consistent presence of the labeled strain at the lettuce

root one week after sowing showed that *Streptomyces* spores could rapidly germinate and produce filamentous mycelium on lettuce. *S. cyaneus* ZEA17I was detected also on two-week-old roots, indicating the long-term survival ability of this strain in lettuce rhizosphere. Altogether, the antagonistic activity, rhizosphere and root competence showed by the *Streptomyces* conferred their potential to act as BCA. Further studies on the complex host-pathogen-antagonist interactions will provide additional knowledge to understand the modes and mechanisms of *Streptomyces*-mediated plant protection.

Key words: *Streptomyces*, biocontrol, lettuce, EGFP (enhanced green fluorescent protein), FISH (fluorescence *in situ* hybridization), CLSM (confocal laser scanning microscopy, colonization.

Introduction

Lettuce

Lettuce (*Lactuca sativa*) is an annual plant in the family *Asteraceae*. It was first cultivated as a food plant by ancient Egyptians by turning it from a weed plant. The English word of "lettuce" was derived from roman "*lactuca*", as the lettuce cultivation was introduced from Egypt to Greece and ancient Rome. According to the Food and Agriculture Organization (FAO), the global production of lettuce in 2010 reached 23,620,000 metric tons (FAO, 2014). Among the countries, China produces more than 50%. Following high production countries are the United States and Italy. However, the cultivars in the United States and European countries are very different from those commonly used in China. The cultivar, *Woju*, also called asparagus lettuce, is widely grown for its stems, and can be eaten either raw or cooked (Kadam and Salunkhe, 1988). Instead, in the United States and Europe, four main botanic types of lettuce are grown: *L. sativa capitata* (L.) Janchen, butterhead lettuce; *L. sativa crispa* L., chrisphead lettuce; *L. sativa longifolia* (Lam.) Janchen, romaine lettuce or cos lettuce; *L. sativa acephala* Dill., leaf lettuce (Mou, 2008). Different cultivars of these lettuce types are grown mainly as salad plants.

The Lombardy region, located in northern Italy, is one of the most important areas of lettuce production in Italy. Lettuce is produced in this area commonly in fields or in greenhouses under plastic tunnels. Farmers sow lettuce in greenhouses and transplant it in the field. Some producers also sow lettuce directly into the field. Leaf lettuce is harvested about 50 days after transplanting, while head lettuce takes up to 75 days. Lettuce is considered as a good source of vitamin A and potassium, as well as a minor source of several other vitamins and essential nutrients (Llorach *et al.*, 2008). Though it is generally grown as a hardy annual plant, and its cultivation is relatively easily, the growth can be negatively affected by numerous nutrient deficiencies, as well as by

plant pathogens (Broadley *et al.*, 2000; Garibaldi *et al.*, 2014). Particularly in Lombardy area, the commercial lettuce cultivation is challenged by several aggressive soil borne fungal pathogens. *Fusarium* wilt of lettuce, which was first reported in eastern Asia and USA (Matuo and Motohashi, 1976; Hubbard and Gerik, 1993), became a serious threat in Italy since 2001 (Garibaldi *et al.*, 2002). The *Fusarium oxysporum* von Schlechtendal can be transmitted by infected seeds (Garibaldi *et al.*, 2004a). Growing lettuce repeatedly every year in the same soil is regarded as one of the main factors contributing to increase the disease severity. Wilt can be observed on young seedlings as the first disease symptom and afterwards, the stunted affected plants develop yellowed leaves and brown or black streaks in the vascular system (Garibaldi *et al.*, 2004b). Root *Verticillium* wilt caused by *Verticillium dahliae* Kleb is another common lettuce soil borne fungal disease (Atallah *et al.*, 2011), widespread all over the world. The pathogen, *Thielaviposis basicola* (Berk & Broome) Ferraris, infesting lettuce roots in the field was reported near Bergamo, Lombardy region (Garibaldi *et al.*, 2005). The disease symptoms included reduced seedling growth, brown-stunted root tissues, and extensive chlorosis that later extended to crown of the plants.

Basal drop, caused by *Sclerotinia* spp., is globally one of the most destructive diseases of lettuce. The disease is widely reported in northern Italy (Bonaldi *et al.*, 2014). *S. sclerotiorum* (Lib.) de Bary and *S. minor* Jagger are the most common causal agents of lettuce basal drop. However, in most cases, only one of the two species is present and causes yield losses in the same location (Van Beneden *et al.*, 2009). Lettuce drop disease symptoms caused by other *Sclerotinia* species were rarely reported. For instance, *S. nivalis* I. Saito was reported to infect and cause basal drop in China (Li *et al.*, 2000). Interestingly, the lettuce cultivar that *S. nivalis* infected was the Chinese one, *Woju*, which is mainly cultivated in China and in some other Asian countries. Lettuce basal drop has a specific epidemiology. The pathogen, *Sclerotinia* spp. can survive in the field as sclerotia in soil for extended periods, as well as mycelium on

dead plant tissues. Sclerotia can infect the lettuce crown, roots, and leaves at any stage of plant development. The hyphae arising from sclerotia penetrate lettuce, directly through senescent leaves and through root tissues, causing wilting and could cause complete plant collapse in less than two days - therefore the appropriate name "*Sclerotinia* Drop". The sclerotia of *S. sclerotiorum* are usually larger than those of *S. minor*. Though they have similar disease cycles, sclerotia of *S. sclerotiorum* can germinate also in shaded areas on the soil surface when moisture is favorable to produce apothecia, which release ascospores into the air for a period of 2 to 3 weeks. These ascospores are carried by air currents and deposited on healthy lettuce plants, which subsequently become infected. The disease cycle of both *S. sclerotiorum* and *S. minor* are described in figure 1 (Subbarao, 1998).

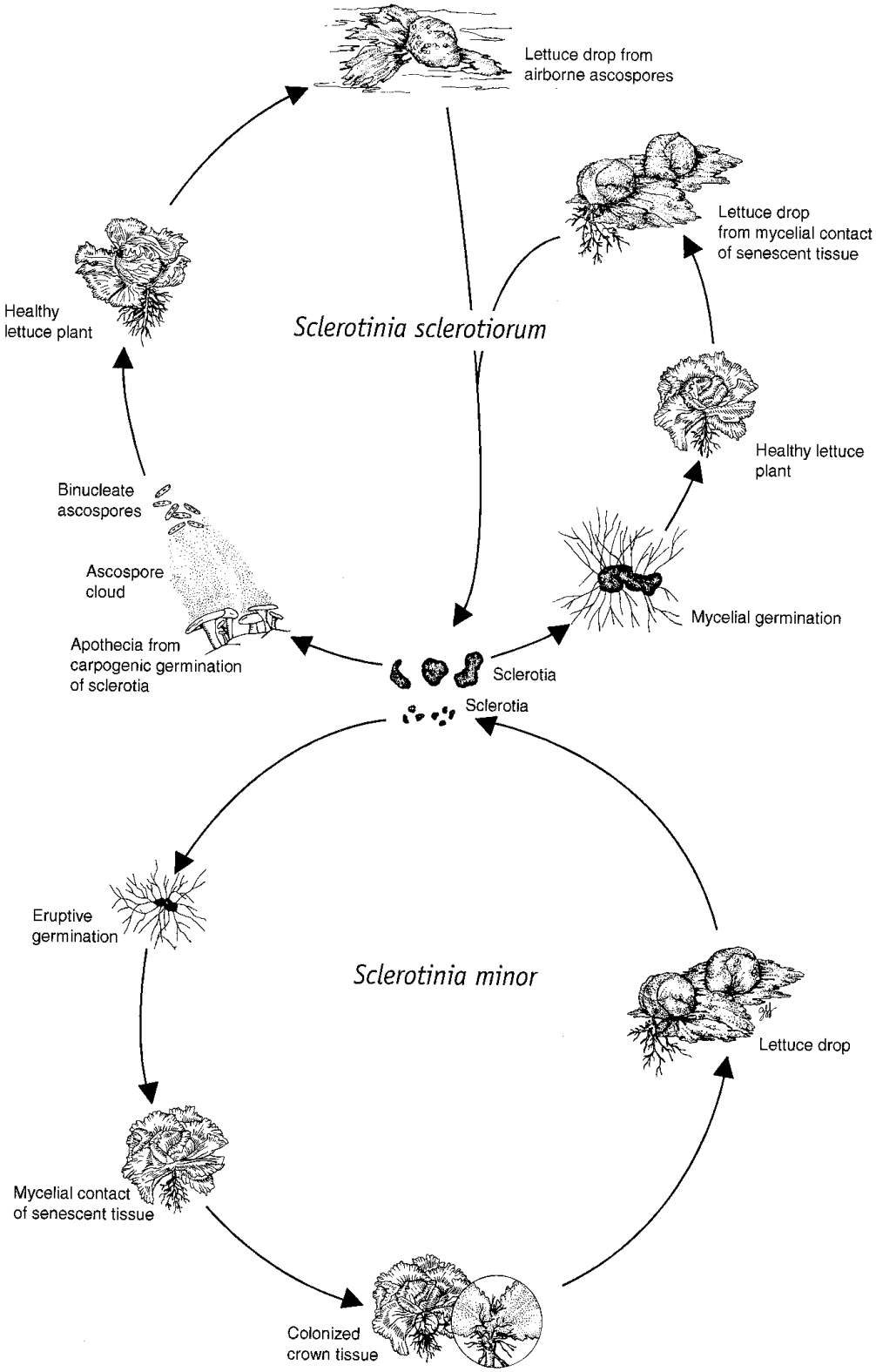


Figure 1. Disease cycle of *Sclerotinia Sclerotiorum* and *S. minor* on lettuce (Subbarao, 1998).

Chemical, cultural, biological, and physical control methods are used to manage soil borne fungal pathogens. Among these, soil solarization is widely used by exploiting the solar energy to increase the soil temperature to levels at which many soil borne fungal plant pathogens would be killed or weakened. Both *Fusarium* wilt and *Verticillium* wilt can be reduced with consistent soil solarization (Chellemi *et al.*, 1997; Tamietti and Valentino, 2001). This method was able to reduce the sclerotia populations of *S. sclerotiorum* in soil, as well as to weaken the ability of the surviving sclerotia to form apothecia (Katan *et al.*, 1987; Phillips, 1990). However, one of the main critical points of this method is its potential impact on soil beneficial microorganisms. Both positive and negative effects on bacterial and fungal populations have been observed (Barbour *et al.*, 2002; Culman *et al.*, 2006; Bonanomi *et al.*, 2008). Despite the effects on microbial community, soil solarization also has a negative impact on bacterivores and fungivores, organisms that feed on bacteria and fungi, and therefore aid in decomposition of soil organic matter, like free-living nematodes (Wang *et al.*, 2006b; Marahatta *et al.*, 2012). Chemical management of soil borne fungal pathogens, including *S. sclerotiorum* on lettuce, was intensively applied. Several fungicides, such as iprodione and vinclozolin showed modest level of lettuce drop control. However, limited by rapid degradation in soil, the ability of iprodione and vinclozolin to control lettuce drop under intensive lettuce cultivation was weak (Chitrampalam *et al.*, 2008). Additionally, resistance to fungicides, including iprodione in *S. sclerotiorum* has been reported under laboratory conditions (Liu *et al.*, 2009). Furthermore, overuse of pesticides resulted in various side effects to human and environment. Meanwhile, higher level of disease control than that provide by fungicides are desired. In the following chapter, I will discuss the significance of the biological control method for pest and disease management, and possible biological control agents that could be used to control soil borne fungal pathogens, including *S. sclerotiorum*.

Towards sustainable agriculture: the application of biological control agents

Food security is one of the current challenges to human development. Plant diseases need to be under control to maintain the quality and abundance of food. The pest management, including plant pathogen control, is becoming more and more important due to massive growth of the human population, as well as global climate change. According to current projections of population growth, the world population will continue to grow until at least 2050, and possibly reach 11 billion by then (Van Den Bergh and Rietveld, 2004). Different strategies and methods could be utilized to prevent, mitigate or control plant diseases. The chemical pesticides have been heavily used in current agriculture, and various side-effects of high chemical input and toxic residue occurrence have been proved a serious threat to environment, human, and other animals (Kohler and Triebskorn, 2013; Lamberth *et al.*, 2013). Except for the negative impact mentioned above, the excessive use of chemical pesticides may have slowed down the genetic improvement of crop yield (de Ponti *et al.*, 2012; Hossard *et al.*, 2014). Nowadays, strict regulations on chemical pesticide use are implemented in all European countries, and there is social pressure to remove the most hazardous chemicals from the market. In the European Union countries, new restrictions for the use of chemicals are being imposed with the new European Directives 2009/128/EC and 1107/2009, which also promote the use of other environmentally friendly methods if they show similar level of control activity (ECPA, 2009). As a result, plant protection scientists are making efforts in developing alternative methods for disease management. One of these possible approaches is biological control (Cook *et al.*, 2007).

The definition of biological control first came with the use of natural enemies to control insect pests (Smith, 1919). In plant pathology, biological control, or its abbreviated synonym biocontrol, applies to the application of microbial antagonists to suppress plant diseases (Handelsman and Stabb, 1996). The first recorded use of a

biological control agent (BCA) can be traced back to the 3rd century in China, where nests of the ant *Oecophylla smaragdina* Fabricius, were commercially sold near Canton to control citrus insect pests such as *Tessaratoma papillosa* Drury (*Heteroptera*) (van Lenteren, 2005). Research and practical application of biocontrol grew very slowly along human history until the introduction of Vedalia beetle, *Rodolia cardinalis* Mulsant, to control cottony cushion scale in 1888 (Altieri, 1991). The general awareness and attention to biocontrol came after 1962, when the Rachel Carson's book "Silent Spring" was published. The book described a situation in the USA, where birds disappeared in spring because of the overuse of chemical pesticides. Accompanied with the hot discussion within human society after the publication of this book, the concept of integrated pest management (IPM) was implemented in the late 1960's (Kogan, 1998). Later, biological control became the core component of IPM (Cook *et al.*, 2007). With the "Silent Spring", biocontrol research also met the spring.

Both in entomology and plant pathology, the organisms used to control insects and pathogens are referred to as the BCAs (Alabouvette *et al.*, 2006; Barratt *et al.*, 2010). An expanded definition of biocontrol has been proposed, in which genetic improvement of the plants and application of natural products extracted or fermented from organisms are considered as biocontrol methods (Tjamos *et al.*, 2010). However, such a wide scope definition caused subsequent debate and it was often considered overbroad by plant protection scientists working in biocontrol (Pal and Gardener, 2006). In more accepted concept, biocontrol is organism-based, and the discovery of new BCA is one of the targets of biocontrol research. The mechanisms of microbial assisted biocontrol may involve: inhibition of the pathogen by producing antimicrobial secondary metabolites; inactivation of pathogen germination factors present in seed or root exudates; degradation of pathogen pathogenic factors such as toxins; competition for iron by production of siderophores; competition for host colonization sites and nutritional resources; induction of plant systemic resistance;

plant growth promotion (PGP); parasitism of pathogens by producing cell wall-lytic enzymes, for example chitinase and β-1,3 glucanase (Fravel, 1988; Emmert and Handelsman, 1999; Suarez-Estrella *et al.*, 2013; Glick, 2015). In practice, different mechanisms may work solely or concurrently, or to be combined without disturbing each other in terms of suppressing plant pathogens (Compant *et al.*, 2005).

Until now, *Coniothyrium*, *Trichodema*, *Bacillus*, and *Pseudomonas* species have been widely studied as BCAs against different pathogens (Walsh *et al.*, 2001; Howell, 2003; Jacobsen *et al.*, 2004). In particular, a *Coniothyrum minitans* W.A. Campb strain was considered the most efficient fungal BCA for the control of white mold of dry bean caused *S. sclerotiorum* under Canadian prairie conditions (Huang *et al.*, 2000). Other *C. minitans* strains also showed potential to control *S. sclerotiorum* on lettuce (Budge *et al.*, 1995). Similarly, disease severity of lettuce drop caused by *S. sclerotiorum* under greenhouse conditions was reduced by treating seedlings with a peat-bran preparation of *Trichodema harzianum* Rifai strain (Inbar *et al.*, 1996). However, other two *Trichodema* strains did not show any effect on disease severity of *S. sclerotiorum* on lettuce, as well as no effect on the survival of the sclerotia (Budge and Whipps, 1991). One *Pseudomonas chlororaphis* Guignard & Sauvageau strain showed similar biocontrol efficiencies to a *Bacillus amyloliquefaciens* Fukumoto strain against stem rot of canola caused by *S. sclerotiorum*. (Fernando *et al.*, 2007). Their efficiencies were comparable to fungicide iprodione application. Two *B. amyloliquefaciens* strains showed activity in suppressing *Sclerotinia* stem rot in soybean through the production of antifungal compounds (Alvarez *et al.*, 2012). The progress towards the sustainable agriculture never stops, and recalls for new biocontrol agents. *Streptomyces* spp. are one of the potential biocontrol agents needed to be understood better, especially the mechanisms involved in pathogen suppression, and interactions with the host and pathogens.

Streptomyces spp. in nature, and their functions in agriculture

Streptomyces are Gram-positive bacteria, ubiquitously found in soil. They are the largest genus of *Streptomycetaceae* family (order *Actinomycetales*), and includes more than 500 species (Takahashi *et al.*, 2002). In soils, *Streptomyces* are responsible for the turnover of organic matter. In other environments, like aquatic systems, and indoor habitats, *Streptomyces* are also widely represented (Johansson *et al.*, 2014; Chen *et al.*, 2015; Rashad *et al.*, 2015). Also agricultural plants harbor abundant streptomycetes. Endophytic streptomycetes were isolated from different plant roots (Sardi *et al.*, 1992; Taechowisan *et al.*, 2003; Cao *et al.*, 2004). Compared to other bacteria, for example, *Escherichia coli* Migula, and *Bacillus subtilis* Ehrenberg (both have approximately 4000 predicted genes), *Streptomyces* have much larger genomes (Kunst *et al.*, 1997). The genome of *Streptomyces coelicolor* A3 (2) encodes around 7950 predicted genes (Bentley *et al.*, 2002). Moreover, they have filamentous and fungi-like life cycle, as shown in Figure 2 (Kieser *et al.*, 2000). The word "*Actinomyces*" comes from Greek and could be translated as "ray fungus" (Waksman and Henrici, 1943). For *Streptomyces*, the production of many secondary metabolites that closely relate to their biological functions, is induced when the aerial hyphae appear and sporulation starts (Hopwood, 1988). *Streptomyces* mutants that were unable to sporulate, were found to be defective also in production of secondary metabolites (Chater K F, 1996; Pope *et al.*, 1996). Thus, sporulation is an important feature involved in the *Streptomyces* biological functions.

Since the 1940s, streptomycetes have been largely exploited in pharmaceutical industry. Early *Streptomyces*-derived antibiotics, Actinomycin, was discovered in 1940, and following Streptomycin was isolated from *Streptomyces griseus* Krainsky in 1943 (Kingston, 2004). After that, a wide group of new antibiotics derived from different *Streptomyces* species has been discovered. One of the newest ones, platensimycin, was isolated from *Streptomyces platensis* Tresner & Backus (Wang *et al.*, 2006a). Currently, *Streptomyces*-derived antibiotics occupy two thirds of the

current antibiotics market (Demain, 2009). Aside antibiotics, diverse beneficial secondary metabolites and enzymes have been identified from *Streptomyces*. For brevity, chitinase produced by streptomycetes was reported to be able to degrade fungal cell walls, where chitin is one of the main components (Gupta *et al.*, 1995; Mahadevan and Crawford, 1997); the production of siderophores, high-affinity iron chelating compounds, is not only responsible for scavenging ferric iron from the environment (Compant *et al.*, 2005), but may also directly inhibit the pathogen growth by iron competition and stirring plant induced systemic resistance (Loper and Buyer, 1991; Audenaert *et al.*, 2002); direct PGP effect was also observed by incubating the plant with streptomycetes able to produce auxin, a plant growth hormone (Yandigeri *et al.*, 2012); additionally, the phosphate solubilization ability of some streptomycetes would hydrolyze organic and inorganic phosphorus from insoluble compounds to increase is availability for plant nutrition (Hamdali *et al.*, 2008; Jog *et al.*, 2014). These properties provide streptomycetes the possibility to be used as potential biocontrol agents, as well as plant growth promotion bacteria (PGPB).

Compared to their huge utilization in medical industry, the application of *Streptomyces* in agriculture still remains hindered. Until now, very few products based on *Streptomyces* as bio-pesticides are available on the market. *MYCOSTOP*®, a *Streptomyces griseoviridis* Anderson strain K 61, formulation product, has been developed and used commercially against various fruit pathogens (Suleman *et al.*, 2002; Velivelli *et al.*, 2014). Another marketed streptomycete bio-pesticide is *Actinovate*®, based on *Streptomyces lydicus* De Boer strain WYEC 108, which also showed activity against different plant pathogens (Handelsman and Stabb, 1996; Matheron and Porchas, 2008). Other *Streptomyces* spp. demonstrating biocontrol activities were reported (Emmert and Handelsman, 1999; Paulitz and Belanger, 2001; Dahiya *et al.*, 2006). Nevertheless, the applications of BCAs often fail under different environmental conditions because of the incomplete knowledge about their interaction

with the host and other natural microflora. The colonization kinetics of the host, such as the plant rhizosphere, by BCAs is a fundamental prerequisite to understand the mechanisms of microbial assisted plant protection and production, especially for less studied BCAs, like *Streptomyces*.

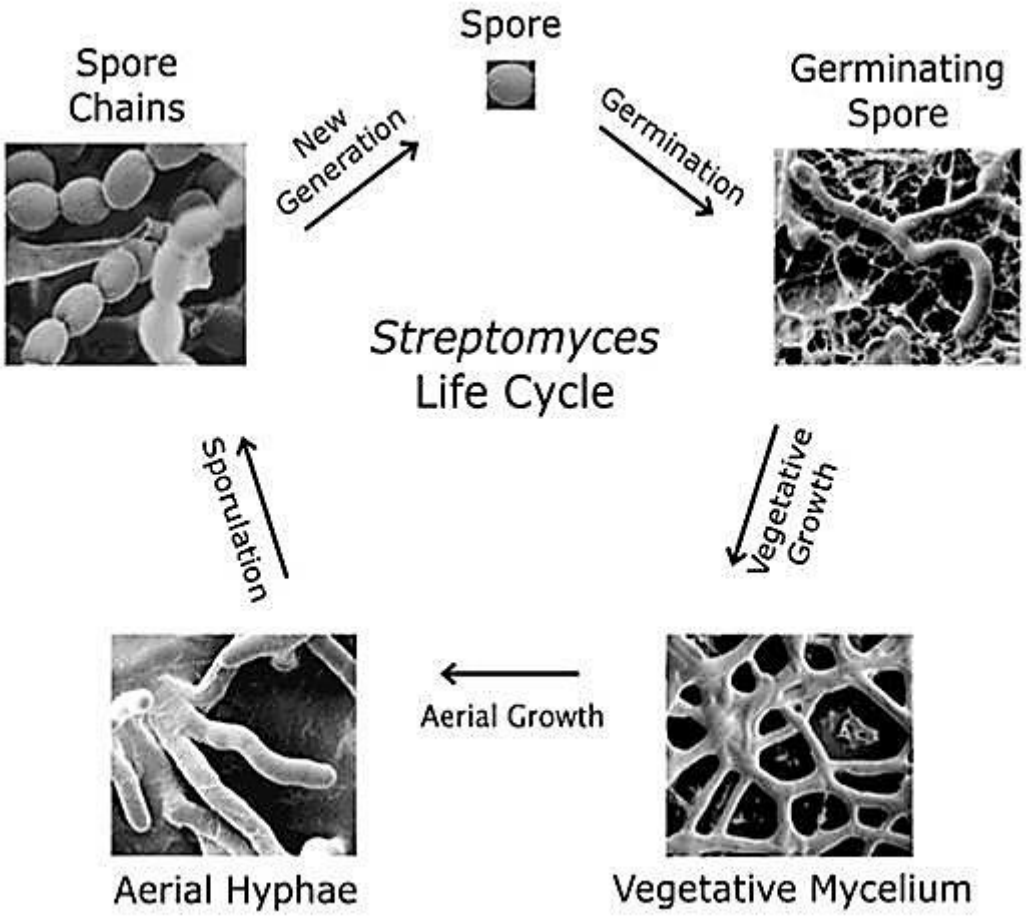


Figure 2. Life cycle of *Streptomyces* (Kieser *et al.*, 2000).

The crucial role of plant colonization by microorganisms in biocontrol mechanisms

It has been widely accepted that significant plant-microbe interactions occurring at specific site usually involve the competence of the microbial colonization. The crosstalk is initiated by the plants producing signals that are recognized by microorganisms. As a reply, the microbes produce signals that induce their plant colonization (Berg and Smalla, 2009). General steps of colonization include attraction, recognition, adherence, invasion (only endophytes and pathogens), colonization, and population growth (Stones and Krachler, 2015). Plant colonizing microorganisms mediate biocontrol through different mechanisms. For example, competition for ecological niches, like specific infection sites by *Trichoderma,* showed protective effect to grapes against *Botrytis cinerea* Pers. *Trichoderma* colonized grape blossom tissue and excluded the pathogen from its infection site (Vinale *et al.*, 2008). Several studies of root colonizing *Trichoderma* showed increased levels of plant enzymes involved in biocontrol, including various peroxidases, chitinases, β-1,3-glucanases, and the lipoxygenase-pathway hydroperoxide lyase (Harman *et al.*, 2004). Moreover, *Trichoderma* strains do not only produce antibiotic metabolites directly, they elicit the plant to produce antimicrobial compounds and contribute to plant induced systemic resistance to pathogens. As an example, root colonization of cucumber by *Trichoderma* strain T-203 led to a significantly higher level of phenolic glucoside in leaves. The carbohydrate moieties removed forms of phenolic glucoside, aglycone, was reported to act as an inhibitor of a wide range of pathogenic bacteria and fungi (Yedidia *et al.*, 2003). Additionally, growth promotion of the plant root system and enhanced nutrient availability upon rhizosphere colonization by *Trichoderma* increased the overall antagonism to plant pathogens (Vinale *et al.*, 2008).

The rhizosphere represents a layer of the soil surrounding the root surface area. This definition was coined more than one hundred years ago (Hiltner, 1904). Rhizosphere

is a complex and dynamic environment harbored by the root that involves diverse microorganisms interactions. The rhizobacteria are the most abundant microorganisms in the rhizosphere microbial community. Rhizobacteria are rhizosphere competent bacteria that colonize well the roots and enhance their survival ability by developing beneficial relationships with the hosts (Hartmann *et al.*, 2009). They demonstrated similar biocontrol mechanisms as *Trichoderma* and other fungal BCAs, but also unique pathways in terms of plant growth and health promotion. They can inhibit the growth of various phytopathogens in a variety of ways like competing for nutrients, niche exclusion, limiting the available iron supply through production of siderophores, and producing anti-fungal metabolites involved in antibiosis, as well as inducing systemic resistance (Bhattacharyya and Jha, 2012). For instance, rhizosphere competent fluorescent pseudomonads showed broad spectrum antagonistic activity against plant pathogens. They must be present at roots in sufficient numbers to have a biocontrol or PGP effect on the plant. For example, to protect plants from *Gaeumannomyces graminis* var. *tritici* Walker or *Pythium* spp. damage, the crucial colonization level of fluorescent pseudomonads must reach 10^5–10^6 CFU (colony-forming units) g^{-1} of root tissue (Haas and Defago, 2005). The fluorescence of fluorescent pseudomonads relies on an extracellular diffusible pigment called pyoverdin (Pvd). It is a siderophore that has high affinity for Fe^{3+} ions. It has been shown that the ability of *Pseudomonas putida* Trevisan strain, B10, to suppress *Fusarium* wilt relies on the siderophore production (Kloepper *et al.*, 1980), while Pvd-negative (Pvd-) mutants showed significantly lower inhibition of phytopathogens (Keel *et al.*, 1989). Later, it was hypothesized that rhizobacteria like fluorescent pseudomonads perform their biocontrol or PGP activity partially by depriving pathogens of iron. Another category of siderophore produced by fluorescent pseudomonads, pyochelin, has been identified as an antifungal compound (Phoebe *et al.*, 2001). Although pyochelin is a relatively weak Fe^{3+} chelator, its good ability to chelate Cu^{2+} and Zn^{2+} may enable it to deprive pathogenic fungi from copper and zinc (Visca *et al.*, 1992). Except for siderophores, biocontrol fluorescent pseudomonads

can produce several other antibiotic compounds. These compounds showed both anti-fungal and anti-bacterial activities (Raaijmakers *et al.*, 2002). Particularly, six of these compounds: phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin, cyclic lipopeptides (all of which are diffusible) and hydrogen cyanide (which is volatile) are well documented against root pathogens. Their antibiosis range and mechanisms were discussed (Haas and Defago, 2005). Up to now, their modes of actions are partially known. For instance, phenazines may catalyze the formation of hydroxyl radicals, which damage lipids and other macromolecules of pathogens (Britigan *et al.*, 1992). Cyclic lipopeptides with surfactant properties were able to insert into cell membranes and lead to antimicrobial activities (Nielsen *et al.*, 2002). Induced systemic resistance is another important biocontrol mechanism of rhizobacteria. Similarly to *Trichoderma*, the induction of plant resistance by colonization with some rhizobacteria, including *Pseudomonas* and *Bacillus*, enhances the plant systemic defense system but does not involve the production of pathogenesis-related proteins (Van Loon, 1997; Vinale *et al.*, 2008). Additionally, instead of targeting specific phytopathogens, induced systemic resistance by rhizobacteria is effective against a range of pathogens (Haas and Defago, 2005).

Many rhizobacteria are able to affect plant growth and development. The PGP rhizobacteria are those bacterial strains that strongly colonize rhizosphere and significantly stimulate plant development (Bloemberg and Lugtenberg, 2001; Lucy *et al.*, 2004; Nelson, 2004; Lugtenberg and Kamilova, 2009). The abilities of rhizobacteria to fix nitrogen, solubilize phosphate, and produce phytohormones (such as auxin and cytokinin) and volatile growth stimulants (such as ethylene and 2, 3-butanediol) contribute to their PGP efficacies. At the same time, the enhanced plant growth, stimulated by rhizobacteria would help plants against biotic and abiotic stress, including phytopathogens. Similar to biocontrol mechanisms, heavy colonization of rhizosphere by rhizobacteria plays a crucial role in their PGP activities (Vessey, 2003).

Among the rhizobacteria, *Streptomyces* species have not been intensively studied compared to *Pseudomonas* and *Bacillus* species. One of the reasons is, as described in previous chapters, that the streptomycetes are still much less used in agriculture than in pharmaceutical industry. They have been long considered simply as free-living soil inhabitants, but their complex interactions with plants and other organisms have being uncovered. Several studies reported rhizosphere competent streptomycetes controlling phytopathogens. *Streptomyces lydicus* WYEC108, which showed stable rhizosphere colonization of pea plants, was able to reduce disease severity of fungal root and seed rot caused by *Pythium ultimum* Trow (Yuan and Crawford, 1995). *Streptomyces. griseoviridis* colonized tomato, and reduced tomato crown rot under field conditions (Nemec *et al.*, 1996). For tomato transplants, *Streptomyces* sp. Di-944 inhibited *Rhizoctonia* causing damping off disease (Sabaratnam and Traquair, 2002). Eight *Streptomyces* strains demonstrated significant effects in suppressing alfalfa and soybean root rot (Xiao *et al.*, 2002). Banana plantlet rhizosphere colonizer, *Streptomyces* sp. strain g10, showed variable rhizosphere population during six weeks, and decreased the final disease severity of leaf symptoms and rhizome discoloration caused by *Fusarium oxysporum* f.sp. *cubense* Snyder & Hansen (Getha *et al.*, 2005). Few species of *Streptomyces* are pathogenic to plants, such as *Streptomyces scabies* Lambert & Loria or *Streptomyces turgidiscabies* Miyajima. They are plant pathogens with broad host range causing important economic losses, especially on tap root sand tuber crops, such as potatoes, sweet potatoes, carrots, or beet. However, other non-pathogenic *Streptomyces* species are good potential inhibitors of these pathogenic species. Antagonistic *Streptomyces* spp. strains colonized well the potato tubers and showed biocontrol efficiency against *S. scabies* causing scab disease (Schottel *et al.*, 2001). Biocontrol of scab on potato tuber caused by *S. turgidiscabies* with nonpathogenic *Streptomyces* spp. strains indicated that complex interactions between species may exist. Although it is well known that streptomycetes are potential BCAs against phytopathogens, even against their own

phyto-pathogenic species, and they could produce various beneficial metabolites and enzymes, their biocontrol mechanisms are not well explored yet.

Rhizosphere competence has a tremendously important role for microorganisms able to suppress soil borne pathogens, as discussed earlier in this chapter. The methodological evolutions, like advanced microscopy and molecular techniques, make it possible to visualize these beneficial microbes to colonize rhizosphere and plant. In the next chapters, currently available approaches and techniques to study the colonization of rhizobacteria will be discussed.

Antibiotic resistance markers as fundamental tools to study microbe colonization dynamics

Several markers have been developed and adapted to study beneficial plant-microbe interactions in the rhizosphere (Bonaldi *et al.*, 2015). Among these, spontaneously occurring antibiotic-resistant mutants of microorganisms have been widely used to study colonization dynamics of microorganisms (Gamalero *et al.*, 2003). Rifampicin resistant mutants were used to study population dynamics of introduced bacteria in the rhizosphere (Mirleau *et al.*, 2000). Furthermore, the stability of rifampicin resistance of *Pseudomonas putida* WCS358 was determined under field conditions (Glandorf *et al.*, 1992). Kanamycin and streptomycin resistance were obtained by Tn*5* mutagenesis with the suicide plasmid method (Simon *et al.*, 1983). The maintenance of Tn*5* in the mutant JM218 was determined by comparing bacterial densities of this mutant in root suspensions, estimated by serological methods, with bacterial density estimated by CFU counting on medium supplemented with kanamycin (Lemanceau *et al.*, 1992; Van Den Bergh and Rietveld, 2004). Despite the use of mutants, various plasmids and transposons have been engineered to study microbe colonization dynamics (VanOverbeek *et al.*, 1997; Mirleau *et al.*, 2001). However, prior to use antibiotic resistance to determine microorganism colonization, the resistance of natural

microflora to the antibiotic used should be verified. Naturally kanamycin-resistant bacteria were found in a Dutch soil up to 2×10^4 CFU per gram of soil (Wilson, 1995). Usually, the level of resistance of the indigenous microflora must be determined prior the study. Moreover, genetic changes associated with chromosomal-mediated antibiotic resistance may results in ecologically important consequences (Gamalero *et al.*, 2003). For instance, Tn5 bleomycin resistance gene confers improved survival growth advantage in *E. coli*, which is a common human pathogen (Blot *et al.*, 1994). In addition, the use of antibiotic-tagged bacteria is accompanied with the risk of antibiotic resistance spreading in nature (Jansson, 1995; Martinez, 2008). However, as a whole, antibiotic resistance approach to study survival dynamics of introduced microbe is quite sensitive, cost effective, reliable and easy to perform.

EGFP marker as a convenient approach to visualize colonization patterns of beneficial microbes

Currently, fluorescent markers, including green fluorescent protein (GFP) are gaining increasing popularity for plant colonization studies. The GFP is a 27 KDa polypeptide which converts the blue chemiluminescence of the Ca^{+2}-sensitive photoprotein (aequorin from the jellyfish *Aequorea Victoria* Murbach & Shearer) into green light (Kremers *et al.*, 2011). Various derivatives of the green fluorescent protein (GFP) have been engineered to increase the fluorescence and to overcome the variable expression of the original marker in different species (Gamalero *et al.*, 2003). Enhanced GFP (EGFP) contains numerous silent nucleotide changes in comparison to GFP to maximize its expression in mammalian cells, and was adopted for use in *Streptomyces* spp., which have a similar codon usage (Haas *et al.*, 1996; Sun *et al.*, 1999). The advantages and disadvantages of GFP have been well discussed (Errampalli *et al.*, 1999). One of the most relevant advantages is that GFP is easily detected, does not require exogenous substrate and allows the monitoring of a single cell. Moreover, it is stable and resistant to proteases. However, several potential

disadvantages may limit the application of GFP in plant colonization studies: the interference with soil particles; the auto-fluorescence of plant tissues that in many cases fluoresce green; the inability to apply in anaerobic conditions; and the instability of the plasmid in different microbes (Haseloff and Siemering, 2005). GFP has been widely utilized to study microbial colonization of roots and rhizosphere under sterile conditions (Prieto *et al.*, 2011; Weyens *et al.*, 2012). Attempts of making advantage of GFP labelled microbes to compete with natural microbial community and to evaluate the competence of the microbes in complex environments were reported (Compant *et al.*, 2008). However, particularly for streptomycetes, their rhizosphere colonization patterns were rarely determined using labeling by fluorescent proteins in combination with confocal laser scanning microscopy (CLSM). Colony development of *Streptomyces antibioticus* Waksman strain, ATCC 11891, and *Streptomyces coelicolor* Muller strain, M145, in soil in the absence of the host plant were examined by CLSM (Manteca and Sanchez, 2009). In their study, chemically fluorescent staining apporach was used, but not application of fluorescent proteins. Similarly, chemically stained pathogenic *S. turgidiscabies* was examined under CLSM to study its survival on radish seedlings (Johnson *et al.*, 2008). However, its further development in the radish rhizosphere was not addressed. Up to now, only the wheat seed colonization by EGFP-tagged *Streptomyces* sp. strain was reported (Coombs and Franco, 2003). Similarly to Johnson et al. (2008), early stage of *Streptomyces* colonization was determined, but long-term rhizosphere competence was not investigated. In addition, horizontal gene transfer, like transforming the GFP gene into a microorganism, may negatively impact on its growth rate, sporulation ability, and secondary metabolite production (Nigro *et al.*, 1999; Lubeck *et al.*, 2002; Weyens *et al.*, 2012). Therefore, the fitness needs to be evaluated to determine the biological activity of the transformed microorganisms before using them for colonization studies.

FISH, a cytogenetic and useful technique to study plant-microbe interactions

Aside fluorescent protein approaches, fluorescence *in situ* hybridization (FISH) is another commonly used technique to study plant colonization by microbes in combination with CLSM. FISH is a cytogenetic technique that uses fluorescent probes that bind to only those parts of the chromosome with a high degree of sequence complementarity (Kliot *et al.*, 2014). The specificity of the probes is defined by their sequence and in most cases these are characteristic signature sequences of rRNA genes. Therefore, FISH is ideal to detect a specific taxa in environmental samples (Amann and Fuchs, 2008). FISH-CLSM has an advantage that it directly visualizes target cells and provides useful estimates of bacterial numbers in certain habitats (Bulgarelli *et al.*, 2012; Cardinale, 2014). Additionally, it also could avoid quantification bias that cultivation or PCR methods may cause (Acinas *et al.*, 2005; Sipos *et al.*, 2010). The relative and absolute abundance of a specific component in a microbial community could be semi-automatically quantified by CLSM and digital image analysis after staining the cells with specific FISH probes (Bouchez *et al.*, 2000; Schmid *et al.*, 2000; Juretschko *et al.*, 2002). Moreover, the localization of the stained cells can be determined microscopically by CLSM (Hofmann *et al.*, 2014; Erlacher *et al.*, 2015). Compared to the fluorescent protein approaches, FISH requires a preliminary fixation. Hence, it is more labor-demanding. In addition, as fixation process kills all cells, live imaging of cells is impossible with FISH. Meanwhile, separated samples fixed at different biological stages do not truly correspond to the time-lapse experiment. Furthermore, the difference between the living and the dead cells cannot be distinguished *in situ* in the biological samples (Cardinale, 2014). This limitation could be a potentially critical point to quantify microbial BCAs or PGPB *in situ*, since most of the considered biological mechanisms of these beneficial microbes are achieved by their living cells. On the contrary, the fluorescent protein approaches

have the advantage that the fluorescent protein gene is only expressed in in living cells, which could be relatively more accurate to reflect the survival of active BCAs or PGPB on plants.

Therefore, both GFP-CLSM and FISH-CLSM have advantages and disadvantages in terms of plant-microbe interaction research, especially for colonization studies. The combination of both techniques could be an ideal choice for comprehensive studies.

Aim of the study

Worldwide, agricultural systems face the challenge of providing for the demand of massively growing human population. To meet this demand, yield losses caused by phytopathogens should be minimized to maintain food quantity and quality. At the same time, yield limitation due to soil fertility and nutrition deficiency add extra pressure to plant production. Thus, searching for sustainable solutions to suppress phytopathogens, as well as to increase the yield is gaining high interests in recent years. A diverse assemblage of plant associated microorganisms can contribute to crucial ecosystem services in agricultural landscapes, including PGP and biocontrol. The beneficial interactions between microorganisms and their host are fundamental prerequisites of microbial assisted plant protection and production. However, comparing to other well studied rhizobacteria, like *Bacillus* and *Pseudomonas* species, the interaction between *Streptomyces* and their hosts are not yet well known, including the colonization of plants by *Streptomyces*.

The objective of this work was to get insight into the colonization of tagged *Streptomyces* strains, selected as potential BCAs, at lettuce roots and rhizosphere. *Streptomyces* strains with potential biological control activity against lettuce drop were EGFP transformed to produce green fluorescence and their fitness were compared to untransformed strains to confirm they maintain their potential as BCAs. The dynamics of *Streptomyces* in lettuce rhizosphere and roots was quantified by a seed coating method with assistance of introduced antibiotic resistance. EGFP-CLSM and FISH-CLSM approaches were applied to study the colonization patterns of *Streptomyces*. The PhD work can be summarized in the following steps:

1. Transformation of the *Streptomyces* strains with the pIJ8641 constructs containing the EGFP and apramycin resistance gene.
2. Evaluation of the fitness of *Streptomyces* strains after genetic transformation.

3. Determining the population dynamics of the *Streptomyces* strains associated with lettuce rhizosphere and surface-sterilized roots.
4. Understanding the localization of lettuce rhizosphere competent Streptomyces strains by assistance of the EGFP-CLSM and FISH-CLSM approaches.

Materials and methods

1. Transformation of the *Streptomyces* strains with the pIJ8641 construct

1.1 Conjugation

Five *Streptomyces* strains, potential BCAs against *Sclerotinia sclerotiorum*, were used in this study: *S. anulatus* CX14W, *Streptomyces* sp. CX16W, *S. exfoliatus* FT05W, *Streptomyces* sp. SW06W, and *S. cyaneus* ZEA17I. They were maintained at the Plant Pathology Laboratory, Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, and selected previously from a wide collection of actinomycetes isolated from roots of different plants (Sardi *et al.*, 1992; Bonaldi *et al.*, 2015). *Escherichia coli* strain ET12567 (harboring the helper plasmid pUZ8002), was provided by prof. Flavia Marinelli, University of Insubria, Italy, and was used as donor strain for conjugation. Plasmid pIJ8641, obtained from prof. Mervyn Bibb, John Innes Centre, UK, was maintained in *E. coli* strain DH5α (Figure 3). It carries the EGFP gene under the constitutive *ermE* promoter, an apramycin resistance marker [*aac*(*3*)*IV*], an *oriT*/RK2 region, and a lambda phage chromosomal integration sequence (Sun *et al.*, 1999). The strain *S. coelicolor* A3(2) was obtained from prof. F. Marinelli, and used as a reference strain to evaluate transformation efficiency. Plasmid pIJ8641 was transformed into the donor strain *E. coli* ET12567 (pUZ8002) by rubidium chloride method (Hanahan, 1983) and conjugated into recipient *Streptomyces* strains as previously described (Kieser *et al.*, 2000). Prior to conjugation, the concentration of the *E. coli* donor strain ET12567 containing plasmid pIJ8641 was adjusted to 1×10^7 CFU/mL. The ex-conjugants were selected on the basis of apramycin resistance. The conjugation efficiency was calculated as number of ex-conjugant colonies per number of recipient spores.

1.2 PCR amplification of the EGFP-transgene

Genomic DNA of wild-type and transformed (EGFP-) strains was extracted by the protocol developed for *Streptomyces* from CTAB method (Kieser *et al.*, 2000). The amplification of 16S rDNA fragment (expected size 1500 bp) was used to evaluate the quality of DNA in all samples, using PCR primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and fD2 (5′-ACGGCTACCTTGTTACGACTT-3′) (Weisburg *et al.*, 1991), and the following thermal cycling conditions: initial denaturation at 94°C for 1 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 30 s and extension at 72°C for 2 min, a final extension at 72°C for 5 min.

PCR primers rEGFP-N (5′-CTGGTCGAGCTGGACGGCGACG-3′) and rEGFP-C (5′-CACGAACTCCAGCAGGACCA TG-3′) were designed to amplify the EGFP gene fragment (expected fragment size 700 bp), using the following thermal cycling conditions: initial denaturation at 94°C for 1 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 2 min, a final extension at 72°C for 2 min. The DNA amplification was carried out using PCR thermal cycler (BioRad, USA), and was performed in a total volume of 25 µL containing 30–50 ng DNA, 0.25 µM each primer, 1 U Go Taq DNA polymerase (Promega, USA), 5 µL of 5x Go Taq buffer (Promega, USA), and 0.2 mM of each dNTP (Invitrogen). The PCR products were visualized under the Gel Doc transilluminator (BioRad, USA) following electrophoresis in 1% (w/v) agarose gel.

1.3 Microscopic observation of EGFP expression

For microscopic observations, transformed (EGFP-) strains were inoculated on Czapek yeast extract agar (CZY; 35 g/L Czapek-Dox Broth Difco, 2 g/L Yeast Extract Difco, 15 g/L agar Applichem). A microscopic cover slide was partially inserted in the medium at the edge of the inoculated strain under a 45° angle to allow the strain to

grow on the cover slide. The plates were incubated at 24°C for 5 days. Subsequently, cover slides were removed from the medium and observed by bright-field and epifluorescence microcopy using Olympus BX51 with the FITC filter set (467–498 nm excitation and 513–556 nm emission) to confirm the expression of EGFP in transformants.

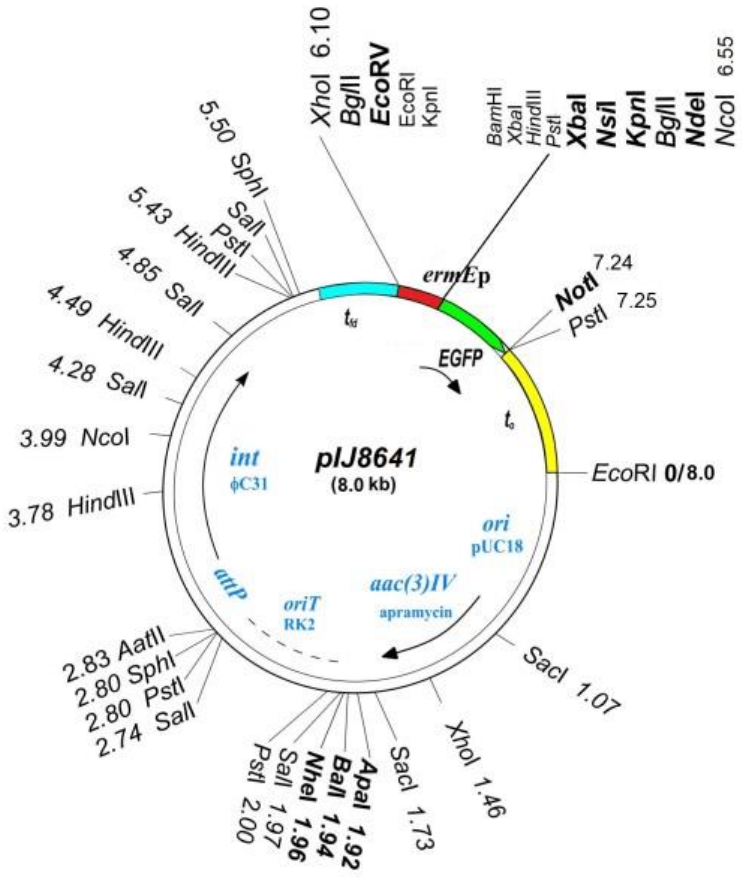


Figure 3. Restriction map of pIJ8641.

2. Impact of the transformation on fitness of *Streptomyces* strains

2.1 Antagonistic activity against *Sclerotinia sclerotiorum*

The antagonistic activity of wild-type and transformed (EGFP-) strains against *S. sclerotiorum* was determined by a dual culture assay on CZY agar (Bonaldi *et al.*, 2014). Briefly, 10 µL of *Streptomyces* spore suspension (1×10^7 CFU/mL) were

inoculated on a 40 mm line two days prior to *S. sclerotiorum* inoculation. An agar-mycelium plug (5 mm diameter), obtained from the edge of an actively growing colony of *S. sclerotiorum* grown on Malt Extract Agar (MEA; 20 g/L Malt Extract, Difco, and 15 g/L agar Applichem), was placed at 25 mm distance from the growing *Streptomyces* colony and the plates were incubated for 72 h at 24°C. Plates inoculated with *S. sclerotiorum* only were used as a control. The antagonistic activity was determined by calculating the percentage of growth inhibition of *S. sclerotiorum* compared to the control. The experiment was repeated twice in three replicates.

2.2 Sporulation

The sporulation of the strains was assessed by plating 1 mL of spore suspension (1×10^7 CFU/mL) on a CZY agar plate and determining the number of spores produced after 6 days of incubation at 30°C (Grantcharova *et al.*, 2005). Five mL of sterile water were added to the Petri plate and the surface of colonies was gently scraped to release the newly formed spores (Kieser *et al.*, 2000). The spore suspension was filtrated through two layers of sterile gauze and the spore concentration (colony formation unite per mL-CFU/mL) was quantified by plating serial dilutions of the spore suspension and counting the number of colonies grown after 4 days of incubation at 30°C. The experiment was repeated twice in three replicates.

2.3 Mycelium biomass growth

The mycelium biomass growth curve of wild-type and transformed (EGFP-) strains was determined daily as follows: 20 µL of *Streptomyces* spore suspension (1×10^7 CFU/mL) were inoculated into a 50 mL tube containing 20 mL of CZY broth, and incubated at 30°C with 200 rpm constant shaking for 8 days. Each day, 2 mL of liquid culture were removed, spun at 10600 g for 10 min and the dry weight of the pellet, expressed in g/L was determined. The experiment was repeated twice in three

replicates.

2.4 Production of secondary metabolites

Indole-3-acetic acid (IAA)

The IAA production of wild-type and transformed (EGFP-) strains was determined as described previously (Bric *et al.*, 1991; Bano and Musarrat, 2003). In brief, 10 µL of the *Streptomyces* spore suspension (1×10^7 CFU/ml) were incubated with constant shaking at 5 *g* in 5 mL CZY broth added with 500 µg/mL tryptophan (Sigma-Aldrich) in the dark at 30°C for 10 days. One mL of the liquid culture was centrifuged for 10 min at 18000 *g*. One mL of the supernatant was mixed with 50 µL 10 mM orthophosphoric acid (Sigma-Aldrich) and 2 mL of Salkowski reagent (1 mL of 0.5M $FeCl_3$ Sigma-Aldrich, in 50 mL of 35% $HClO_4$ Sigma-Aldrich). The tubes were incubated at room temperature for 30 min. The development of pink color indicated the IAA production, which was quantified by spectrophotometer (Eppendorf) at 530 nm. The experiment was repeated twice in three replicates.

Siderophores

Ten mL of Fe-free Czapek solution (300 g/L $NaNO_3$, 50 g/L KCl, 50 g/L $MgSO_4 \cdot 7H_2O$) were mixed with 15 g/L agar, 30 g/L sucrose, 1 g/L K_2HPO_4, and 5 g/L yeast extract to prepare the Fe-free Czapek agar medium. 10 µL of *Streptomyces* spore suspension (1×10^7 CFU/mL) were inoculated in the center of a Fe-free Czapek agar plate and incubated at 30°C for two weeks. Subsequently, the *Streptomyces* colony was overlaid by 15 mL of the Chrome azurol S (CAS) agar (Schwyn and Neilands, 1987; Perez-Miranda *et al.*, 2007). The siderophore production was determined as a color change in the overlay medium (from blue to orange) after 24 h of incubation at room temperature. The experiment was repeated twice in three replicates.

Chitinase

The colloidal chitin and the colloidal chitin agar were prepared from chitin power (Acfa Aesar) as described previously (Saima *et al.*, 2013). Ten µL of *Streptomyces* spore suspension (1×10^7 CFU/mL) were inoculated in the center of colloidal chitin agar plate (chitin as single carbon sources) as a 40 mm line and incubated at 30°C for 10 days. The production of chitinase was determined based on the presence of a clear hydrolysis zone on the agar plate below the colony. The experiment was repeated twice in three replicates.

Phosphate solubilization

The phosphate solubilization activity of wild-type and transformed (EGFP-) strains was assessed using a plate assay with National Botanical Research Institute's Phosphate (NBRIP) medium, in which $Ca_3(PO_4)_2$ is the only phosphate source (Nautiyal, 1999). Ten µL of *Streptomyces* spore suspension (1×10^7 CFU/mL) were inoculated in the center of a NBRIP-medium Petri plate and incubated at 30°C for 2 weeks. The phosphate solubilization ability was determined based on the presence of a clear hydrolysis zone on the agar plate below the colony. The test was repeated twice in three replicates.

3. Lettuce colonization dynamics by two tagged *Streptomyces* strains

Fifty ice queen lettuce seeds (*Lactuca sativa* var. *capitata*, Iceberg group, Semeurop, Italy) were sterilized in 1 ml of 0.7% sodium hypochlorite (NaOCl) for 5 minutes and were rinsed three times with sterile water. Ten µL of *Streptomyces* spore suspension (EGFP-ZEA17I or EGFP-SW06W) (1×10^8 CFU/mL) were added to the surface-sterilized seeds, and incubated under the laminar flow hood until dry. Another 50 non-inoculated seeds were used as control. Thirty seeds of each treatment were sown

individually in 200cc plastic cups containing 8cm of two-times autoclaved sterile sandy substrate ("Sabbia Vagliata" Gras Calce s.p.a., Italy) and incubated and incubated in a growth chamber (24°C, 55% relative humidity and 15 h photoperiod). The cups were watered daily with 10 ml of sterile water. Ten ml of 10-times diluted KNOP solution (1 liter contains 500mg/L $Ca(NO_3)_2$, 125mg/L $MgSO_4$, 125mg/L KNO_3, 125mg/L KH_2PO_4, 1mg $FeCl_3$) were added instead of sterile water twice a week, on Monday, and Friday.

Six plants arising from bacterized and control seeds were collected at two-, four- and six weeks. Seedlings with the whole root system were carefully taken off the growth substrate and the bulk soil was removed by gently shaking the plants.

3.1 Lettuce bulk soil colonization

For the bulk soil analysis, the entire amount of bulk soil was collected and weighed. The substrate was mixed and divided in two identical parts. One part was incubated in the oven at 50°C and the dry weight was determined. The other part was stirred in sterilized water (1:2 substrate fresh w/v) for one hour and serial dilutions were plated on water-agar medium (WA) containing 15 g/L agar, Applichem, and four antibiotics (25 mg/L nalidixic adic Sigma-Aldrich, 50 mg/L apramycin Sigma-Aldrich, 50 mg/L nystatin Carlo Erba, and 50 mg/L cycloheximide Sigma-Aldrich). Plates were incubated for 7 days at 24°C and *Streptomyces* colonies were counted. The *Streptomyces* population dynamics was expressed as CFU/g of growth substrate dry weight.

3.2 Lettuce rhizosphere colonization

For the rhizosphere analysis, each seedling was cut at base and the roots were vortexed two times for 15 s in 3-5 mL (volume varying according to period of

sampling) of sterilized 0.9% NaCl Sigma-Aldrich, and 0.02% Silwet L-77 Chemtura (washing solution) in Falcons. The roots were removed and used for inner root tissue analysis described below. The suspension was filtered through a 300 μm nylon mesh settled on the top of another Falcon, following a 60 s quick centrifuge to exclude rest of water on the nylon mesh. The rhizosphere soil was collected and its dry weight was determined. The suspension was centrifuged at 10600 *g* for 10 min and the pellet was resuspended in 0.5–1.5 mL of washing solution and plated in serial dilutions on WA medium with four antibiotics mentioned above. The plates were incubated at 24°C for 7 days. *Streptomyces* colonies were counted and the concentration was expressed as CFU/g of rhizosphere dry weight.

3.3 Lettuce inner-root colonization

For inner root tissues analysis, the roots were surface sterilized with propylene oxide for certain time (two-week-old plants, 20 mins; four- and six-week-old plants, 1 hour). Then, they were washed in washing solution (two-week-old plants, 1.5 mL; four- and six-week-old plants, 2.5 mL) and 0.5 mL of the total volume was plated on WA medium to verify the absence of contaminants. Subsequently, the roots were finely homogenized in washing solution and left to macerate for one hour and the suspension was plated on WA medium with four antibiotics in serial dilutions. The *Streptomyces* concentration was determined as described before and expressed as CFU/g of roots dry weight.

4. CLSM observation of lettuce root colonization by *S. cyaneus* EGFP-ZEA17I

Prior to colonization studies of lettuce roots by *S. cyaneus* EGFP-ZEA17I, the overnight pure culture of the strain, grown on CZY agar medium, was observed by confocal laser scanning microscopy (CLSM) to reconfirm the EGFP expression.

Then, a plant colonization assay was prepared, in which the lettuce root colonization by EGFP-ZEA17I was observed at CLSM at different time-points: 2-, 3-, and 14 days after inoculation (dai).

The lettuce seeds were sterilized and bacterized with EGFP-ZEA17I as described before. Afterwards, nine bacterized seeds were planted in a 0.5 cm depth with sterile tweezers into a seed tray filled with 640 g of a mixture of autoclaved quarz sand (Scherf GmbH & Co. KG, Austria) and peat soil ("Gramoflor Profi-Substrat-Topfpikier M+Ton+Fe" GBC, Kalsdorf, Austria) in 1:3 ratio (w/w), and 200 ml sterile tap water. In two trays, no seeds were planted to monitor the soil moisture (≥ 25 %) by a moisture analyzer (MB35 Halogen, Ohaus, USA). Nine surface sterilized non-bacterized seeds were planted into seed trays prepared in the same way as described above and were used as control. After the planting, the seed trays were incubated in a growth chamber (24°C, 55% relative humidity and 14 h photoperiod).

Two- and three-day-old plants were harvested to monitor early colonization of lettuce by EGFP-ZEA17I. Furthermore, fourteen-day-old plants were used to verify the ability of EGFP-ZEA17I to colonize lettuce for longer period. For each observation, three bacterized plants and one non-bacterized plant (negative control) were taken out of the seed tray with a sterile spatula and put into a petri dish. Each sample was taken from a different seed tray. The roots of each plant were freed from the soil and separated from the phyllosphere by using a sterile scalpel, and then cut into 0.5 cm long sections to be directly observed under CLSM. Samples were observed with a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) equipped with solid state lasers for excitation. Filter settings were adjusted to achieve the maximum signal from EGFP and low background autofluorescence of the plant tissues. The EGFP was excited with a 488 nm laser beam and the detection window was optimized for every field of view, in order to gain a better discrimination between the signals and the noise. Plant tissues were excited with a 635 nm laser

beam and the autofluorescence emitted in the range 650–690 nm was recorded. The fluorescent signals from EGFP and from plant tissues were acquired sequentially. For each field of view, maximum projections of an appropriate number optical slices were acquired with a Z-step of 0.15–0.5 µm ("confocal stacks") and the software Imaris 7.3 (Bitplane, Zurich, Switzerland) was used for post-processing, 3D rendering and creation of isosurface-spot models (Erlacher *et al.*, 2015).

5. CLSM observation in combination with fluorescence in situ hybridization (FISH)

Similarly to the EGFP approach, two-, three-, and fourteen-day-old root samples were harvested from different seed trays. For each observation, three lettuce samples inoculated with *S. cyaneus* ZEA17I and one lettuce sample from the negative control were fixed for the fluorescence in situ hybridization (FISH). The fixation was done according to (Cardinale *et al.*, 2008). Shortly, root samples were fixed with paraformaldehyde overnight, and washed four times with cold Phosphate-buffered saline (PBS) solution and then stored at -20°C in 1:1 (vol : vol) 96% ethanol: PBS until FISH-CLSM analysis. FISH-CLSM analysis was performed using specific probes following the protocol by (Cardinale *et al.*, 2008). Briefly, after pre-treatment with lysozyme, the bacteria-universal probe EUB338MIX - an equimolar mixture of Cy3-labelled EUB338, EUB338II and EUB338III probes (Amann *et al.*, 1990) - was used for the detection of bacteria and a Cy5-labelled *Streptomyces* genus specific probe, Strepto (Stackebrandt *et al.*, 1991), was applied for the detection of *S.cyaneus* ZEA17I. Sequences of the probes and the formamide concentration (FA %) used for each probe were listed below (Table 1). Cy3, and Cy5 labeled FISH probes were sequentially excited with 532-, and 635-nm laser beams, respectively; the emitted light was detected in the ranges of 500 to 540 nm, 550 to 610 nm, and 650 to 700 nm, respectively. After hybridization, the samples were washed at 44°C for 15 mins in washing buffer. The FISH stained samples were then mounted with SlowFade Gold

Antifadent (Molecular Probes, Eugene, OR, USA) and stored at 4°C overnight until confocal images acquisition using a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems GmbH, Mannheim, Germany) as described above (Erlacher *et al.*, 2015).

Table 1. Probes used in the FISH experiment of this study.

Name	**Sequence (5'–3')**	**Target**	**FA %**[1]	**Reference**
EUB338	gctgcctcccgtaggagt	Most bacteria	10	(Amann *et al.*, 1990)
EUB338II	gcagccacccgtaggtgt	*Planctomycetales*	10	(Daims *et al.*, 1999)
EUB338III	gctgccacccgtaggtgt	*Verrucomicrobiales*	10	(Daims *et al.*, 1999)
Strepto	gcgtcgaattaagccaca	*Streptomyces*	50	(Stackebrandt *et al.*, 1991)

[1]FA %, formamide concentration.

6. Statistical analysis

All analyses were done using R software, version R3.0.2 (R Core Team, 2014). The statistical differences between EGFP-transformed and wild-type strains in terms of *S. sclerotiorum* growth inhibition, mycelium growth rate, sporulation, and IAA production were compared by a Student's *t*-test ($P = 0.05$). The percent data were arcsine root-square transformed. The soil, rhizosphere and root colonization data were log-transformed and submitted to ANOVA, followed by a Tukey *post hoc* test for multiple comparison ($P = 0.05$), using the Tukey C package (Faria, 2013).

Results

1. Transformation of *Streptomyces* strains with the pIJ8641 construct

1.1 Conjugation

All six strains, including the reference strain *S. coelicolor* A3(2), were transformed with the pIJ8641 plasmid harboring the EGFP gene under a constitutive promoter and apramycin resistance. The conjugation efficiency was calculated as the number of ex-conjugant colonies divided by the number of recipient spores. Conjugation efficiency varied among strains. The highest conjugation efficiency - 3.13×10^{-5} CFU/recipient strain - was obtained for the strain *Streptomyces* sp. SW06W. In general, four strains, *S. anulatus* CX14W, *Streptomyces* sp. SW06W, *Streptomyces* sp. CX16W, and *S. exfoliatus* FT05W showed conjugation efficiency similar to *S. coelicolor* A3 (2), while one strain, *S. cyaneus* ZEA17I conjugated with lower efficiency, equal to 5.81×10^{-8} CFU/recipient strain (Table 2).

Table 2. Conjugation efficiencies of five *Streptomyces* spp. strains and reference strain *S. coelicolor* A3(2) with the pIJ8641 plasmid.

Strain	**Recipient strain (CFU/mL)**	**Conjugation efficiency (CFU/recipient strain)**
S. coelicolor A3(2)	1×10^{8}	9.10×10^{-6}
S. anulatus CX14W	1×10^{8}	4.64×10^{-5}
Streptomyces sp. CX16W	1×10^{10}	6.88×10^{-6}
S. exfoliatus FT05W	1×10^{9}	1.60×10^{-6}
Streptomyces sp. SW06W	1×10^{8}	3.13×10^{-5}
S. cyaneus ZEA17I	1×10^{9}	5.81×10^{-8}

1.2 PCR amplification of the EGFP-transgene

Genomic DNA of both wild-type and transformed *Streptomyces* strains was extracted, and the PCR amplification was performed to verify the presence of the EGFP fragment to confirm successful transformation. The EGFP fragment (700 bp), was detected in all transformed strains by the PCR amplification (Figure 4a). The 16S rDNA fragment (1500 bp), was amplified in all samples, both wild-type strains and transformed strains (Figure 4b). No fragment was detected in the negative control of H_2O (Figure 4ab).

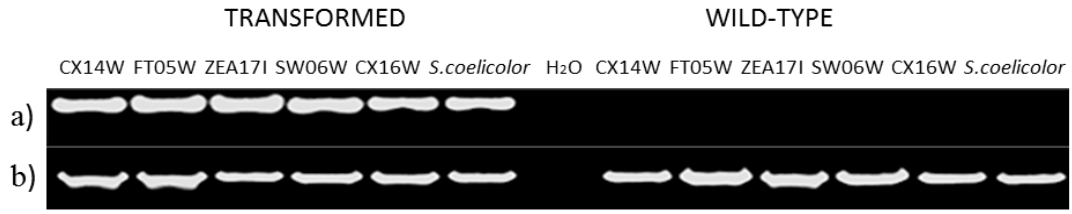


Figure 4. PCR amplification of the EGFP and 16S rDNA fragments. a) PCR to detect EGFP gene fragment, 700 bp. b) PCR to detect 16S rDNA fragment, 1500 bp. H_2O was used as negative control.

1.3 Microscopic observation of EGFP expression

The expression of EGFP was confirmed by epifluorescence microscopy observation. The transformed (EGFP-) strains showed consistent green fluorescence upon exposition to fluorescent light (Figure 5), while the corresponding wild-type strains did not fluoresce (not shown). For instance, filamentous mycelium and spore chains of EGFP-ZEA17I showed strong fluorescence (Figure 5a), as well as those of EGFP-FT05W (Figure 5b). Spore chains of EGFP-CX16W also showed bright green fluorescence (Figure 5c).

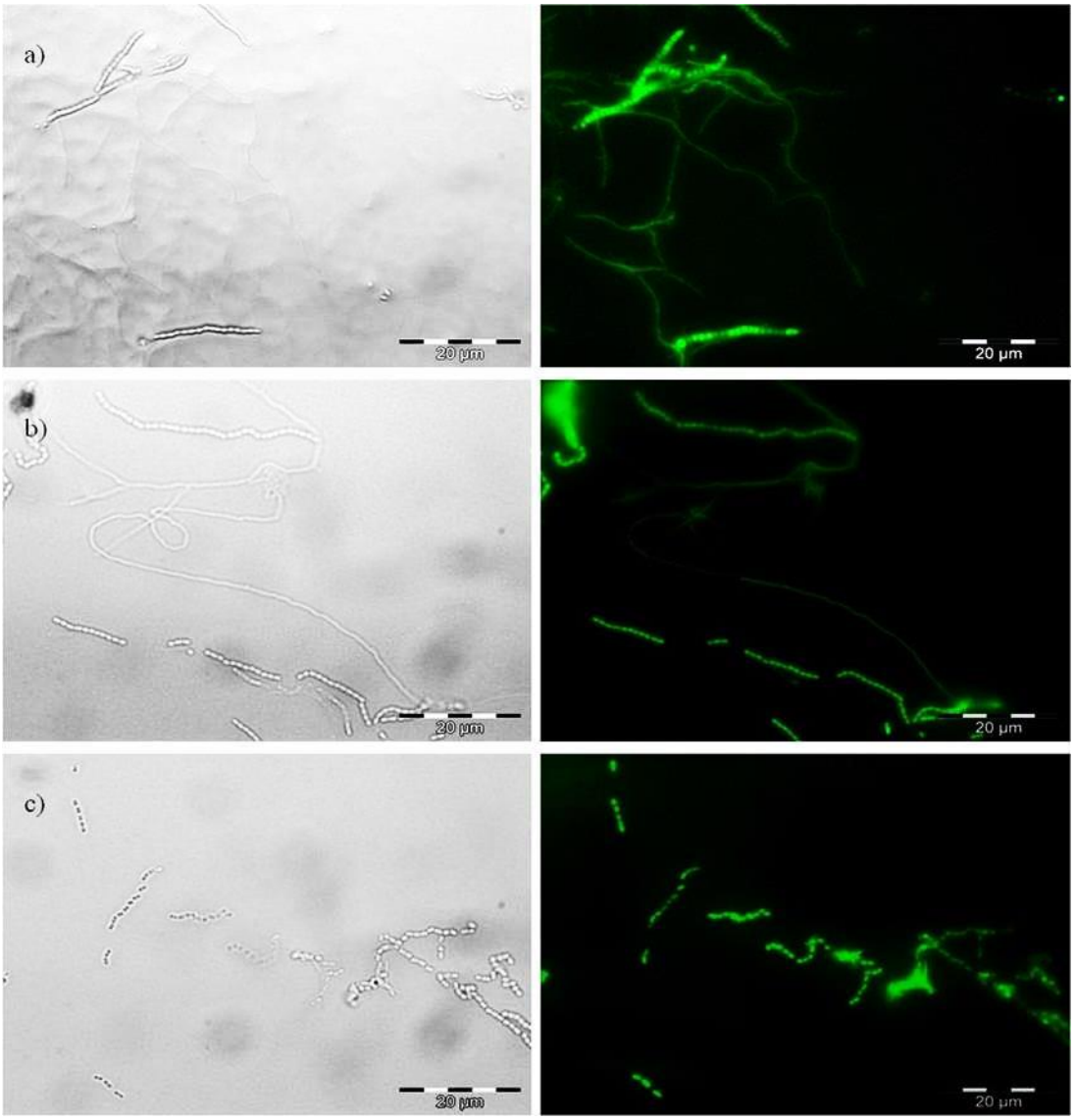


Figure 5. Microscopic observation of EGFP expression in transformed *Streptomyces* strains by bright field (left) and epifluorescence microscopy (right). Mycelium and spore chains of (a) the strain *S. cyaneus* EGFP-ZEA17I, (b) the strain *S. exfoliatus* EGFP-FT05W, and (c) spore chains of the strain *Streptomyces* sp. EGFP-CX16W. Scale bar, 20 µm.

2. Impact of the transformation on fitness of *Streptomyces* strains

2.1 Antagonistic activity

Following the transformation, the inhibition of *S. sclerotiorum* mycelium growth was evaluated. The antagonistic activity of wild-type strains against *S. sclerotiorum* ranged from 67.57% to 80.21%; the highest activity among wild-type strains was in SW06W, while the lowest antagonism was in FT05W. After the conjugation, all transformed (EGFP-) strains inhibited *S. sclerotiorum* radial growth ranging from 65.87 to 80.75%, which were similar to their corresponding wild-type strains. The highest antagonism of EGFP-strains was in EGFP-ZEA17I; and the lowest inhibition activity was in EGFP-CX16W (Table 3).

Table 3. *In vitro* antagonistic activity of transformed (EGFP-) strains against *Sclerotinia sclerotiorum*, compared to their corresponding wild-type strains.

Strain	***S. sclerotiorum* inhibition (%)**	
CX14W	78.80±1.70[1]	
		P^2=0.072
EGFP-CX14W	73.00±2.28	
CX16W	68.99±0.98	
		P=0.091
EGFP-CX16W	65.87±1.33	
FT05W	67.59±0.68	
		P=0.684
EGFP-FT05W	67.13±0.85	
SW06W	80.21±1.34	
		P=1.000
EGFP-SW06W	80.21±1.34	
ZEA17I	74.80±1.76	
		P=0.069
EGFP-ZEA17I	80.75±1.51	

[1] Mean values followed by SE, [2] *P*-values of the Student's-*t* test pairwise comparisons.

2.2 Sporulation

The sporulation of wild-type strains ranged from 0.47 to 7.47 (10^9 CFU/ml); the highest sporulation among wild-type strains was in CX14W; while the lowest sporulation was in FT05W. After transformation, all the transformed (EGFP-) strains showed similar sporulation ability to their corresponding wild-type strains (Table 4); they sporulated from 0.78 to 6.80 × 10^9 CFU/ml; the highest sporulation of EGFP-strains was in EGFP-CX14W and the lowest sporulation was in EGFP-SW06W.

Table 4. Sporulation of transformed (EGFP-) strains, compared to their corresponding wild-type strains.

Strain	**Sporulation (10^9 CFU/ml)**	
CX14W	7.47±3.22[1]	
		P^2=0.229
EGFP-CX14W	6.80±4.91	
CX16W	4.23±6.58	
		P=0.178
EGFP-CX16W	5.55±1.61	
FT05W	1.45±3.69	
		P=0.242
EGFP-FT05W	1.37±4.56	
SW06W	2.27±1.15	
		P=0.205
EGFP-SW06W	0.78±1.67	
ZEA17I	0.47±0.23	
		P=0.417
EGFP-ZEA17I	1.63±1.15	

[1] Mean values followed by SE, [2] *P*-values of Student's-*t* test pairwise comparisons.

2.3 Mycelium biomass growth

The mycelium growth (biomass weight, g/L) was assessed after transformation up to 8 dai (days after inoculation). All the transformed (EGFP-) strains showed similar mycelium biomass to their corresponding wild-type strains (Figure 6abcd, Table 5) at each dai. There were small differences of the peak of mycelium biomass growth between different coded strains. For instance, EGFP-CX16W and CX16W, EGFP-ZEA17I and ZEA17I, and EGFP-SW06W and SW06W reached the maximum mycelium growth at 4 dai (Figure 6bcd), while EGFP-CX14W and CX14W had the peak at 7 dai (Figure 6a). As an example, mycelium biomass growth of EGFP-FT05W and its corresponding wild-type strain FT05W was shown in details at each dai (Table 5). The highest biomass of EGFP-FT05W reached 3.99 g/L at 4 dai, as well as of FT05W, which was 3.90 g/L at 4 dai. After that, both EGFP-FT05W and FT05W started to decrease mycelium biomass growth, and reached 1.95 g/L and 2.18 g/L respectively at at 8 dai. There was no significant difference between EGFP-FT05W and FT05W at each dai.

Tabel 5. Mycelium biomass growth of *S. exfoliatus* EGFP-FT05W, compared to its wild-type strain.

Strain	**Mycelium biomass growth (g/L)**					
	1dai[1]	2dai	3dai	4dai	7dai	8dai
FT05W	2,76±0.45a[3]	3.09±0.31a	3.64±0.49a	3.90±0.39a	2.16±0.63a	2.18±0.43a
EGFP- FT05W	2.61±0.54[2]a	2.94±0.29a	3.65±0.33a	3.99±0.68a	2.25±0.59a	1.95±0.36a

[1] days after inoculation. [2] Mean values followed by SE. [3] Student's-*t* test pairwise comparisons, means in a column with same letters were not significantly different (*P*=0.05).

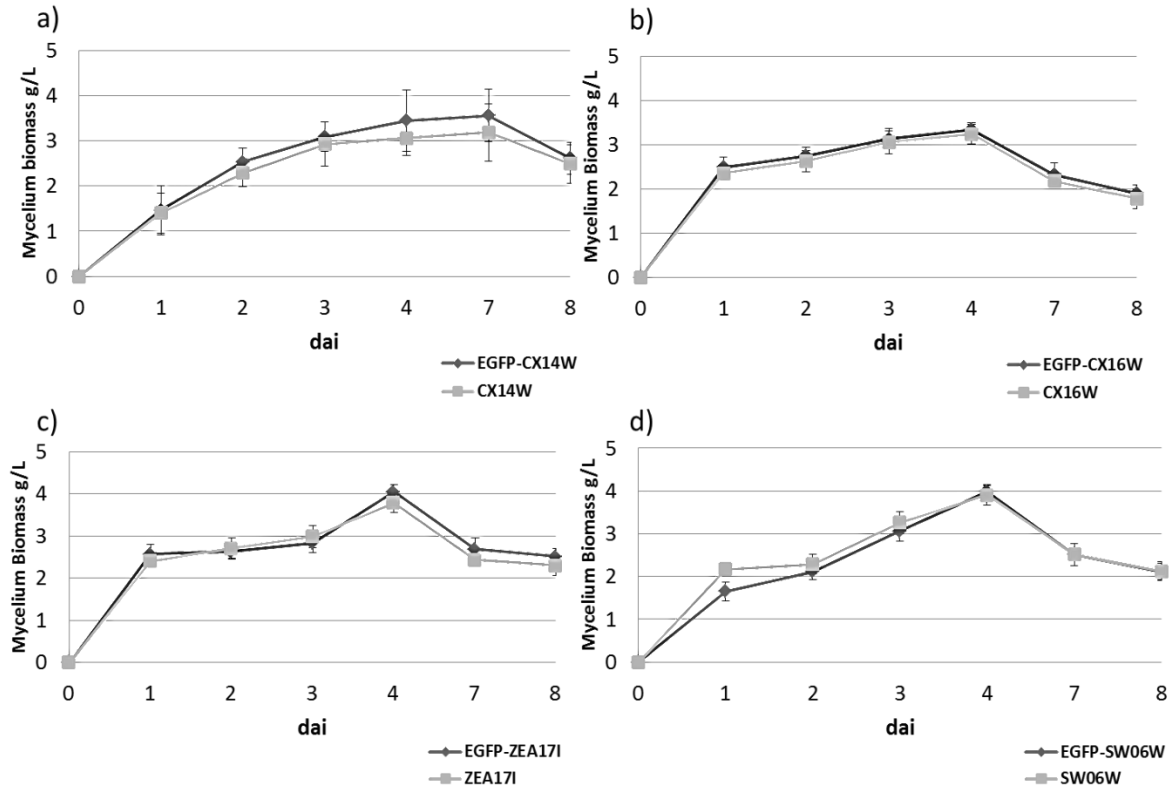


Figure 6. Mycelium growth of the wild-type and its corresponding transformed (EGFP-) strains, (a) CX14W, (b) CX16W, (c) ZEA17I and (d) SW06W. Vertical bars represent SE (N = 3).

2.4 Production of secondary metabolites

Indole-3-acetic acid (IAA)

All the transformed (EGFP-) strains were able to synthesize IAA, ranging from 5.11 to 7.32 µg/ml; EGFP-SW06W produced the highest IAA amount among EGFP-strains; the lowest synthesis was in EGFP-CX14W. There were no statistically significant differences between the EGFP-strains and their corresponding wild-type strains. The wild-type strains could syntheses 5.85 to 7.56 µg/ml; the highest value of in ZEA17I, and the lowest one was in CX14W (Table 6).

Table 6. Indole-3-acetic acid synthesis in transformed (EGFP-) strains, compared to their corresponding wild-type strains.

Strain	**Indole-3-acetic acid (IAA) (µg/ml)**	
CX14W	5.85±0.44[1]	
		P^2=0.229
EGFP-CX14W	5.11±0.35	
CX16W	5.87±0.86	
		P=0.178
EGFP-CX16W	6.03±0.24	
FT05W	6.04±0.60	
		P=0.242
EGFP-FT05W	7.11±0.21	
SW06W	7.32±0.66	
		P=0.205
EGFP-SW06W	6.88±0.28	
ZEA17I	7.56±0.59	
		P=0.417
EGFP-ZEA17I	6.84±0.38	

[1] Mean values followed by SE, [2] *P*-values of Student's-*t* test pairwise comparisons.

Phosphate solubilization, chitinase, and siderophore production

All the wild-type strains were able to solubilize phosphate and produce chitinase, as well as their corresponding transformed (EGFP-) strains. On the contrary, siderophore production was only detected in CX14W and CX16W. Their EGFP-strains, EGFP-CX14W and EGFP-CX16W maintained the abilities in siderophore production (Table 7).

Table 7. Phosphate solubilization, chitinase, and siderophore production of the wild-type and transformed (EGFP-) *Streptomyces* spp. strains.

Strain	Chitinase production	Phosphates solubilization	Siderophore production
CW14W	+	+	+
EGFP-CW14W	+	+	+
CW16W	+	+	+
EGFP-CW16W	+	+	+
FT05W	+	+	-
EGFP-FT05W	+	+	-
SW06W	+	+	-
EGFP-SW06W	+	+	-
ZEA17I	+	+	-
EGFP-ZEA17I	+	+	-

$^{+}$ indicates activity. $^{-}$ indicates no activity.

3. Lettuce colonization dynamics by two tagged *Streptomyces* strains

The colonization dynamics of lettuce bulk soil, rhizosphere, and inner-root tissue by two transformed (EGFP-) *Streptomyces* strains, EGFP-ZEA17I and EGFP-SW06W were evaluated in sterile sandy substrate by seed coating method. Rhizosphere/bulk soil (rhizo/bulk) proportion was also determined to compare the distribution of *Streptomyces* in lettuce rhizosphere and growth substance.

3.1 Bulk soil colonization

Two weeks after inoculation, the EGFP-ZEA17I strain was recovered at 3.33 x 10^1 CFU/g dry weight of substrate and the amount remained stable in the bulk soil up to six weeks at 1.03 × 10^2 CFU/g dry weight of substrate. Meanwhile, the EGFP-SW06W showed similar behavior to EGFP-ZEA17I in lettuce bulk soil, which was

stable from two to six weeks at the amount of 3.0 x 10^1 - 1.4 x 10^2 CFU/g dry weight of substrate (Table 8). There was no significant difference in the survival in bulk soil between the two strains at any of the re-isolation times (Figure 7).

Table 8. Population dynamics of two transformed (EGFP-) *Streptomyces* strain in lettuce bulk soil, CFU g/soil dry weight.

Strain	**Bulk soil (CFU/g dry weight)**		
	2 weeks	**4 weeks**	**6 weeks**
EGFP-ZEA17I	3.33×10^1ns[1]	8.43×10^1ns	1.03×10^2ns
EGFP-SW06W	3.00×10^1ns	7.10×10^1ns	1.40×10^2ns

[1] Tukey post hoc test, means in a row with same letters were not significantly different (P = 0.05).

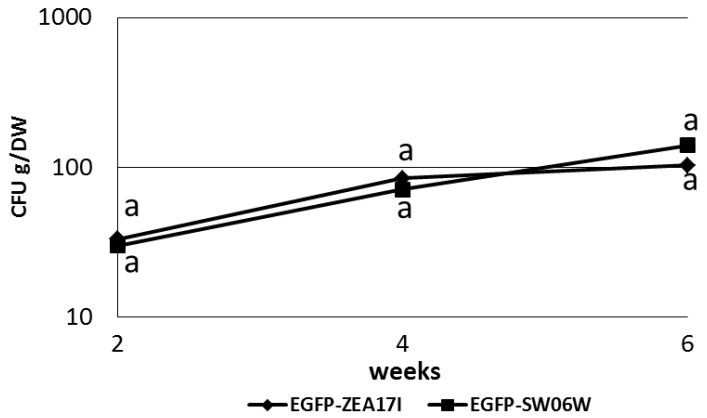


Figure 7. Comparison of population dynamics between two transformed (EGFP-) *Streptomyces* strains in lettuce bulk soil, CFU g/soil dry weight. Means at the same time point with same letters were not significantly different (P = 0.05).

3.2 Lettuce rhizosphere colonization

Both EGFP-ZEA17I and EGFP-SW06W showed stable population dynamics in lettuce rhizosphere from two to six weeks after sowing, ranging from 1.35×10^5 to

2.37×10^4 and from 3.07×10^5 to 7.23×10^4 CFU/g rhizosphere dry weight, respectively (Table 9). However, statistical significance between two weeks and six weeks were observed in EGFP-SW06W. More colonies were recovered from two-week-old lettuce rhizosphere. For EGFP-ZEA17I, there were no statistical differences between all the three re-isolation time-points mean values. Interestingly, there were no differences between two strains at two-week-old lettuce rhizosphere and four-week-old lettuce rhizosphere, but EGFP-SW06W showed higher competence at six-week-old lettuce rhizosphere than EGFP-ZEA17I (Figure 8).

Table 9. Population dynamics of each transformed (EGFP-) *Streptomyces* strain in lettuce rhizosphere, CFU g/rhizosphere dry weight.

Strain	**Rhizosphere (CFU/g dry weight)**		
	2 weeks	**4 weeks**	**6 weeks**
EGFP-ZEA17I	1.35×10^5ns[1]	5.29×10^4ns	2.37×10^4ns
EGFP-SW06W	3.07×10^5a	8.43×10^4ab	7.23×10^4b

[1]Tukey post hoc test, means in a row with same letters were not significantly different (P = 0.05).

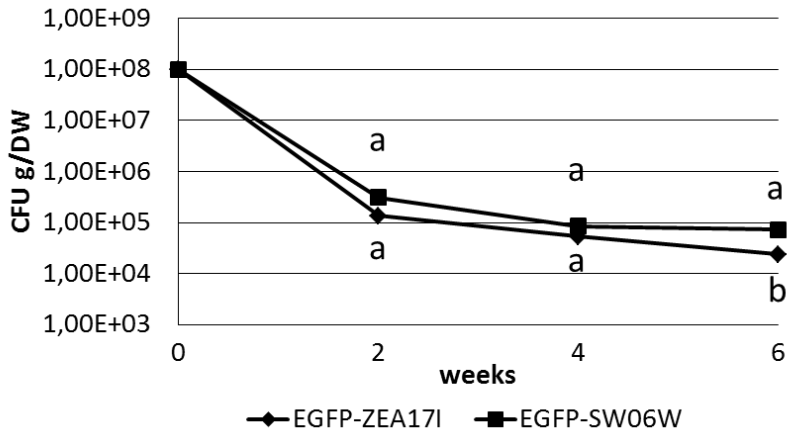


Figure 8. Comparison of population dynamics of two transformed (EGFP-) *Streptomyces* strains in lettuce rhizosphere, CFU g/soil dry weight. Means at the same time point with same letters were not significantly different (P = 0.05).

3.3 Rhizosphere/bulk soil proportion

Both EGFP-ZEA17I and EGFP-SW06W colonized to a higher extent the lettuce rhizosphere than the bulk soil, but the ratio of rhizosphere/bulk soil (rhizo/bulk) decreased after six weeks. For EGFP-ZEA17I, significantly lower rhizo/bulk proportion was observed for six-week-old plants compared to two-week-old plants. For EGFP-SW06W, two-week-old plants showed significantly higher rhizo/bulk proportion than four- and six-week-old plants; and there was no significant difference between four- and six-week-old plant (Table 10).

Table 10. Rhizosphere and bulk soil proportions of two transformed (EGFP-) strains, CFU g/rhizosphere dry weight.

Strain	**Proportion (Rhizosphere/bulk soil)**		
	2 weeks	**4 weeks**	**6 weeks**
EGFP-ZEA17I	4.01×10^{3}a[1]	6.27×10^{2}ab	1.97×10^{2}b
EGFP-SW06W	1.02×10^{4}a	1.19×10^{3}b	5.15×10^{2}b

[1]Tukey post hoc test, means in a row with same letters were not significantly different ($P = 0.05$).

3.4 Lettuce inner-root colonization

In lettuce inner-root tissue, up to six weeks, both EGFP-ZEA17I and EGFP-SW06W demonstrated consistent colonization kinetics from 8.24×10^{5} to 5.81×10^{4} and 6.31×10^{5} to 2.93×10^{4} CFU/g fresh weight of root tissue, respectively. For each strain, there was no statistical significance observed between different isolation times (Table 11). Interestingly, similar to the rhizosphere, EGFP-SW06W showed significant higher colonization at six-week-old inner root tissue of lettuce than EGFP-ZEA17I (Figure 9).

Table 11. Population dynamics of two transformed (EGFP-) *Streptomyces* strains in lettuce inner-root tissues, CFU g/root tissue fresh weight.

Strain	**Inner root (CFU/g fresh weight)**		
	2 weeks	**4 weeks**	**6 weeks**
EGFP-ZEA17I	8.24×10^{5}ns[1]	1.53×10^{5}ns	5.81×10^{4}ns
EGFP-SW06W	6.31×10^{5}ns	2.93×10^{5}ns	3.11×10^{5}ns

[1] Tukey post hoc test, means in a row with same letters were not significantly different (P = 0.05).

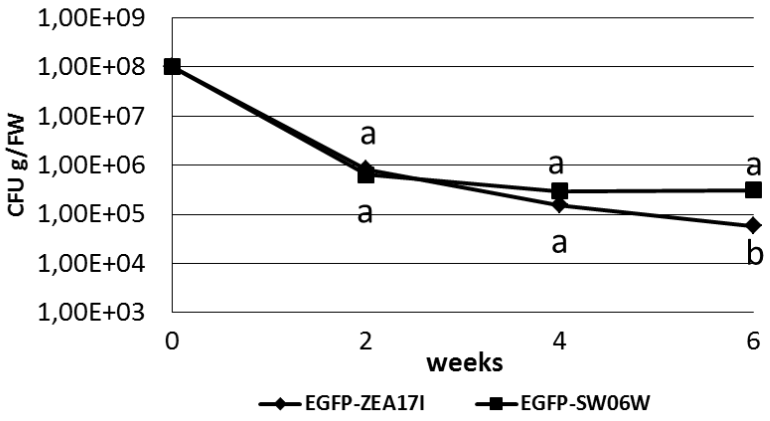


Figure 9. Comparison of population dynamics of two transformed (EGFP-) *Streptomyces* strains in lettuce inner-root tissue, CFU g/root tissue fresh weight. Means at the same time point with same letters were not significantly different (P = 0.05).

4. CLSM observation of lettuce colonization by EGFP-ZEA17I

4.1 One-week-old plant

Filamentous growth of EGFP-ZEA17I on lettuce root system one week after plant sowing was frequently observed (Figure 10). For instance, Mycelium of EGFP-ZEA17I heavily colonized one area of lettuce root surface (Figure 10a). At the root

hair zone of lettuce, filamentous EGFP-ZEA17I was observed closely attached to the hairs (Figure 10b). Except for colonization lettuce root by its mycelium, germinated spores of EGFP-ZEA17I were found in an area which was close to the root hair zone (Figure 10c). Interestingly, a lettuce root tissue where a piece of soil substrate remained attached to the outer root, allowed us to observe that EGFP-ZEA17I colonized more extensively and abundantly the lettuce root than the soil particle (Figure 10d).

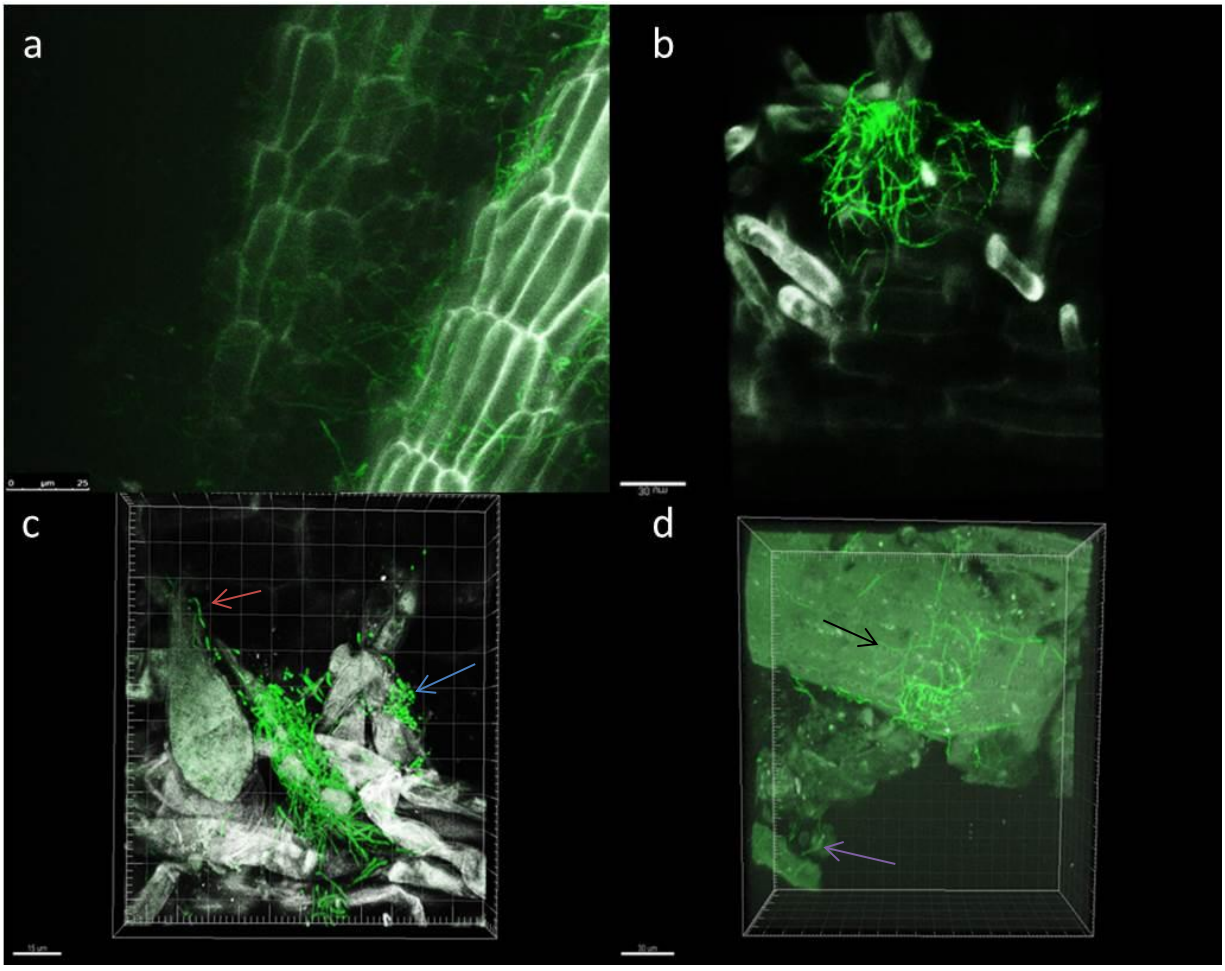


Figure 10. CLSM observations of lettuce root colonization by EGFP-ZEA17I, within one week after bacterization. Filamentous colonization at (a) root surface, scale bar, 25 μm (b) root hair zone, scale bar, 30 μm (c) area close to the root hair zone, red arrow pointed to single hypha, and blue arrow pointed to a group of germinated spores, scale bar, 15 μm (d) root tissue with soil subtract attached, black arrow pointed to the mycelium on root which were more abundant than on the soil particle, which was purple arrow pointed to, scale bars, 30μm.

4.2 Two-week-old plant

On two-week-old lettuce roots, we also observed *Streptomyces* colonization by EGFP-ZEA17I. *Streptomyces* from different stages of its life cycle appeared concurrently at some sites of lettuce root tissues. For instance, spores, single hypha, spore chains, and groups of mycelium of EGFP-ZEA17I were detected on the root surface (Figure 11 and Figure 12).

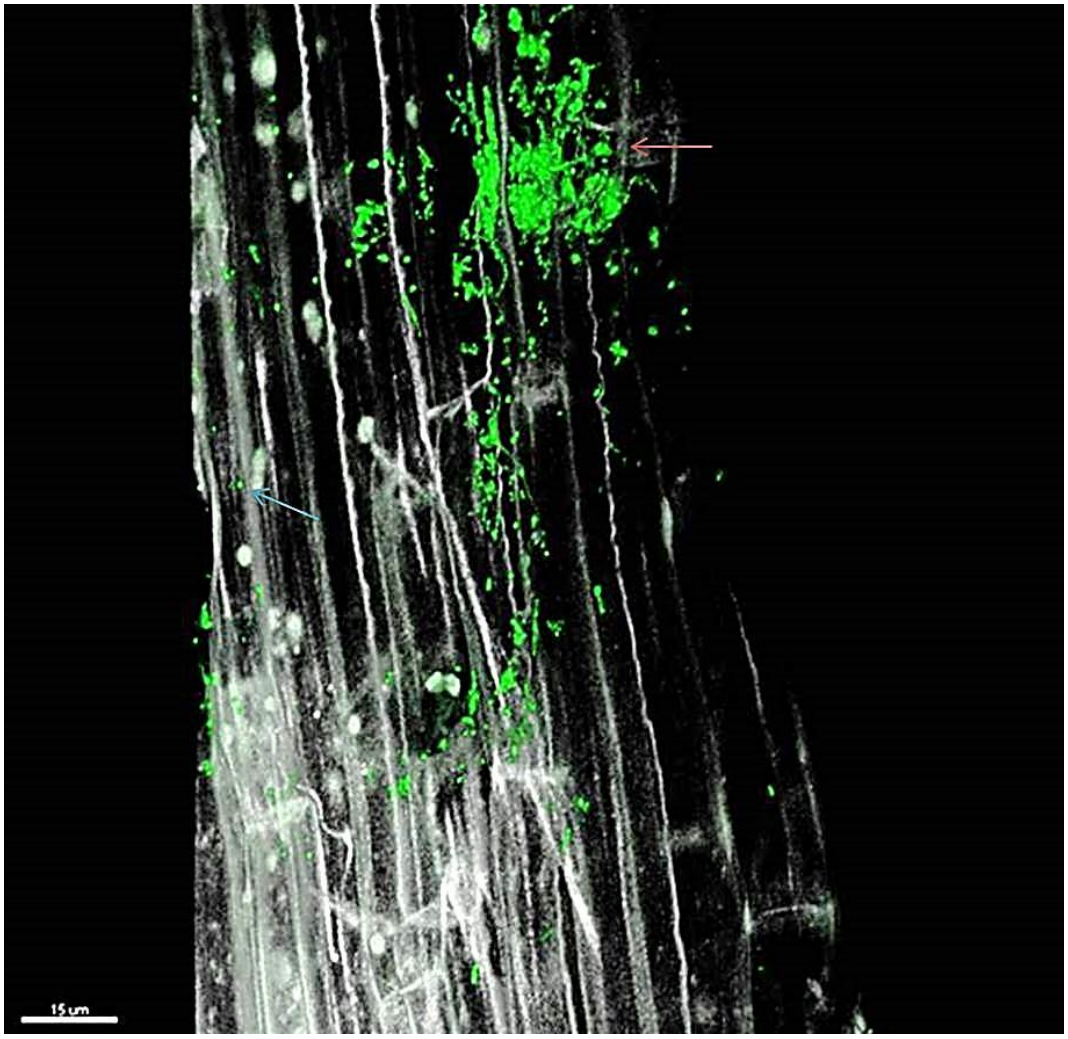


Figure 11. CLSM observations of lettuce root sample A colonized by EGFP-ZEA17I, two weeks after bacterization. Blue arrow pointed to a spore, and red arrow pointed to groups of mycelium, scale bar, 15μm.

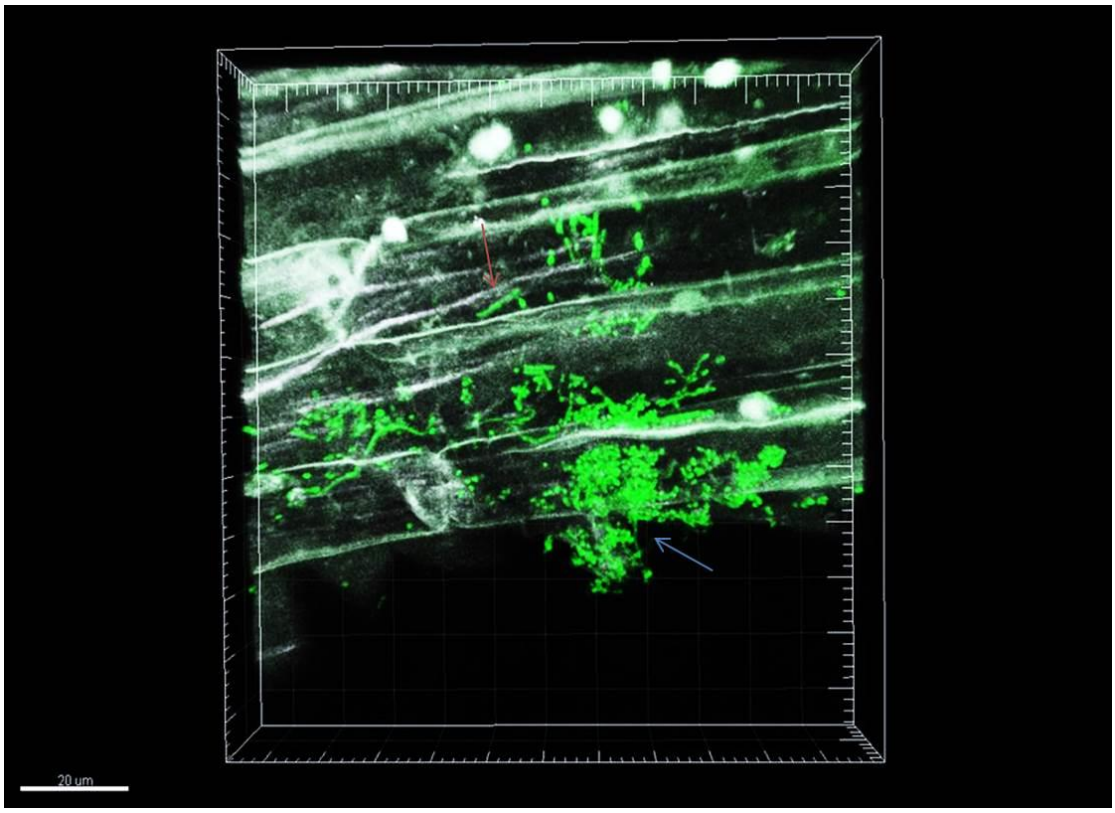


Figure 12. CLSM observations of lettuce root sample B colonized by EGFP-ZEA17I, two weeks after bacterization. Blue arrow indicated a mycelium group, and red arrow pointed to a shore chain, scale bar, 20μm.

5. CLSM observation of lettuce colonization by EGFP-ZEA17I in combination with FISH

5.1 One-week-old plant

Colonization of lettuce root by EGFP-ZEA17I was re-confirmed by FISH-CLSM approach. Both hybridizations of Cy3-labelled EUB338MIX probe (red fluorescence), and Cy5-labelled Strepto probe (green fluorescence) worked in EGFP-ZEA17I. *Streptomyces* confocal photos acquired simultaneously under both channels (Cy3 and Cy5) exhibited yellow fluorescence (Figure 13). EGFP-ZEA17I spores rapidly germinated and started filamentous mycelium growth on lettuce within one week after sowing in soil (Figure 13a). Large mycelium group was detected along the lettuce root tissue (Figure 13b). Interestingly, in the lettuce hair zone, a single EGFP-ZEA17I hypha was observed that colonized a single lettuce hair (Figure 13cd).

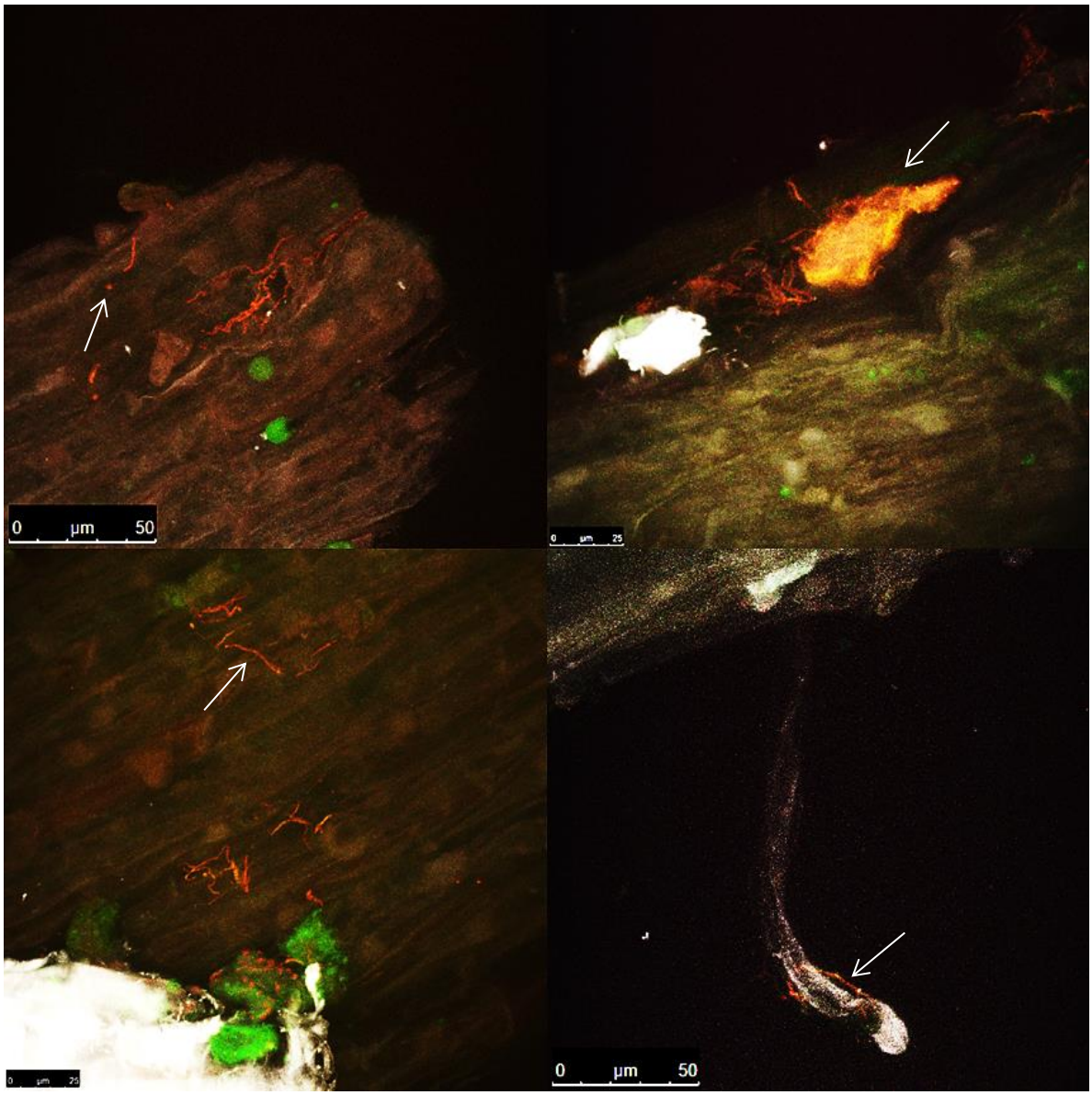


Figure 13. CLSM-FISH observations of lettuce root colonization by EGFP-ZEA17I one week after bacterization at four sites. (a) arrow pointed to a germinated spore, scale bar, 50µm, (b) arrow pointed to a large mycelium group, scale bar, 25µm, (c) arrow pointed to a single hyphae, scale bar, 25µm, (d) arrow indicated to a single hyphae colonizing lettuce root hair, scale bar, 50µm.

5.2 Two-week-old plant

EGFP colonization on lettuce two-week-old root was also re-confirmed by CLSM in combination with FISH. Similar to what was observed on one-week-old plant, a large EGFP-ZEA17I "colony", mycelium groups, was found at one site of lettuce root tissue (Figure 14, shown by green fluorescence signal of *Streptomyces* specific probe acquired under the Cy-5 channel). A clear and typical spore chain of *Streptomyces* was also observed on another lettuce root tissue, where single spore was also detected (Figure 15, shown by green fluorescence signal of *Streptomyces* specific probe acquired under the Cy-5 channel).

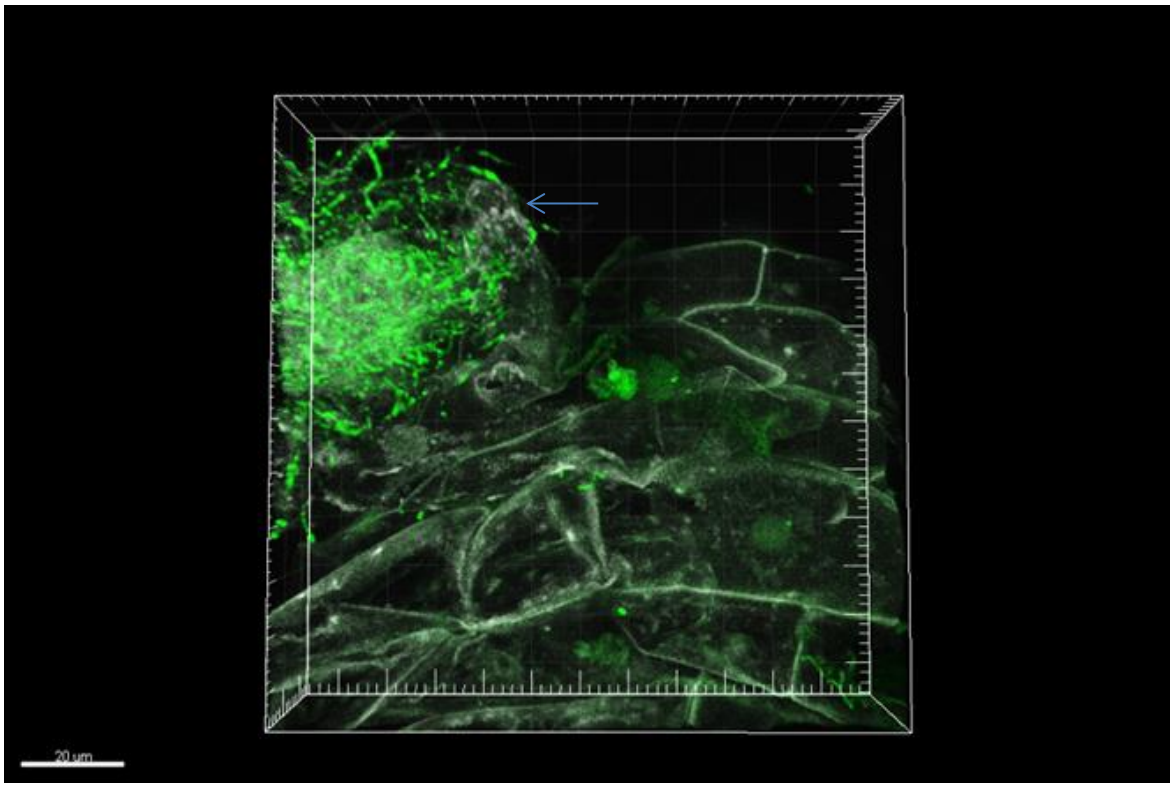


Figure 14. CLSM-FISH observations of lettuce root sample C colonized by EGFP-ZEA17I two week after bacterization. Blue arrow pointed to a large *Streptomyces* colony, scale bar, 20 µm.

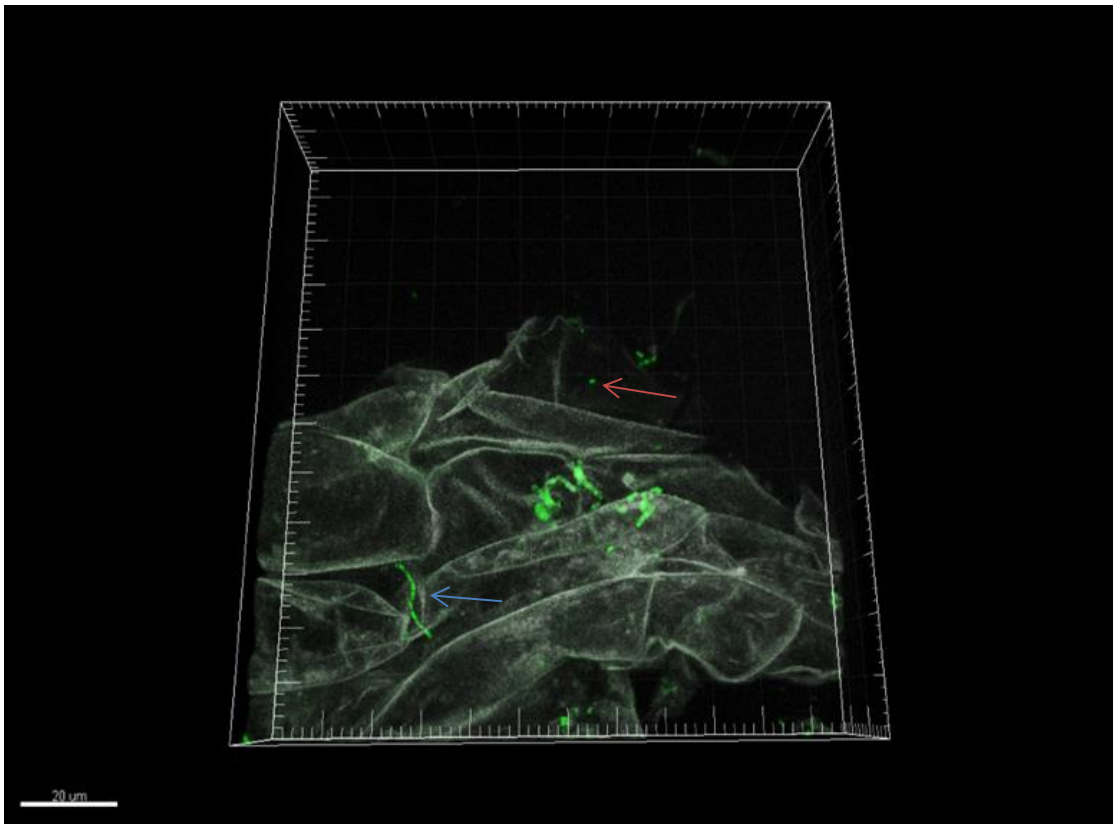


Figure 15. CLSM-FISH observations of lettuce root sample D colonized by EGFP-ZEA17I two week after bacterization. Blue arrow pointed to a typical spore chain of *Streptomyces*, and red arrow pointed to a single spore, scale bar, 20μm.

Discussion

Plant associated microbes can contribute to crucial agro-ecosystem services. Indeed, successful control of phytopathogens and enhanced plant growth by application of these beneficial microorganisms are increasingly reported (Paulitz and Belanger, 2001; Nelson, 2004). However, their large scale applications in field are often limited, also due to insufficient knowledge about their interactions with the host in the rhizosphere. Therefore, understanding the colonization of plant rhizosphere and root system by these microorganisms is gaining high interest nowadays in order to develop sustainable solutions for agriculture, like new BCAs and PGPB (Lugtenberg and Kamilova, 2009; Compant *et al.*, 2010).

The aim of the PhD work was to investigate the colonization of lettuce roots and rhizosphere by tagged *Streptomyces* spp. with biological control potential, to better understand if and how they inhabit the lettuce rhizosphere and colonize plant roots. Five *Streptomyces* strains were selected on the basis of their strong *in vitro* antagonism activity against one of the major lettuce soil borne pathogens, *S. sclerotiorum* (Bonaldi *et al.*, 2014). They were transformed with the pIJ8641 plasmid harboring apramycin resistance marker and EGFP gene under a strong constitutive promoter (Sun *et al.*, 1999). The conjugation efficiency varied, and for most strains it was comparable to the reference strain *S. coelicolor* A3 (2). The pIJ8641 plasmid integrates at the chromosomal attachment site for the temperate phage φC31, which may result in disruption or alteration of fitness and biological activity of the transformed strains. Indeed, decrease or loss of biological activity was reported after GFP-transformation of various BCAs, such as *Pseudomonas putida* Trevisan, *Metschnikowia pulcherrima* Pitt & Miller, or *Clonostachys rosea* Schroers (Nigro *et al.*, 1999; Lubeck *et al.*, 2002; Weyens *et al.*, 2012). Following the transformation, we compared several biological parameters important for biological control and PGP

between transformed and wild-type strains, before studying their interactions with the host plant. None of the transformed strains showed altered growth or sporulation, which could have conferred a disadvantage in plant root and rhizosphere colonization. Also, all the transformed strains retained the ability to suppress growth of *S. sclerotiorum in vitro*. Therefore they will also be used for studying their interactions with the pathogen and the mechanisms of biological control *in vivo*. Moreover, we compared the expression of some of the most common traits involved in PGP and biological control (Brader *et al.*, 2014), such as production of auxins, siderophores and lytic enzymes, and no change in performance between the wild-type and transformed strains was observed.

We chose two strains, EGFP-ZEA17I and EGFP-SW06W, which showed the highest antagonism against *S. sclerotiorum in vitro* among the transformed strains, for pilot studies of lettuce roots and rhizosphere colonization. We adopted seed coating method to deliver *Streptomyces*, and evaluated their population dynamics in rhizosphere, bulk soil, and inner-root tissues of lettuce, grown in double sterilized sandy substrate. We quantified the population dynamics of the tagged strains by exploiting the introduced apramycin resistance. Both strains were able to survive in the bulk soil up to six weeks after inoculation, although at low quantity, 10^1 - 10^2 CFU/g dry weight. Different from the bulk soil, both strains showed high competence up to six weeks in the lettuce rhizosphere in the range of 10^4 - 10^5 CFU/g dry weight. The proportion of rhizosphere/bulk soil of the two strains slightly decreased when compared the values of six-week-old plants to two-week-old plants. It could be hypothesized that streptomycetes multiply the population in bulk soil faster than the increase in rhizosphere after six weeks. To determine the ability of two strains to colonize lettuce inner-root tissues, we verified that no *Streptomyces* colonies were recovered from the surface of root tissue after sterilization. Similar to the rhizosphere, both strains showed stable colonization also at the inner-root tissue in the quantity of 10^4 - 10^5 CFU/g fresh weight. For each strain, there were no significant differences between

time points: two-, four-, and six weeks. Interestingly, in both lettuce rhizosphere and inner-root tissues of the six-week-old plants, EGFP-SW06W showed higher competence than EGFP-ZEA17I. These results indicated that EGFP-SW06W may colonize better the lettuce rhizosphere and inner-root tissue for long-term period under the conditions we used.

Studying the dynamics of colonization by other beneficial microorganisms exploits the strain identification mostly by natural or introduced antibiotic resistance and its quantification by dilution plating (Gamalero *et al.*, 2003; Gamalero *et al.*, 2005). We made advantage of the apramycin resistance marker of the pIJ8641. Both strains colonized lettuce rhizosphere and inner-root tissue in high quantities up to six weeks, which was close to half of the lettuce cultivation cycle. The high proportion of rhizosphere/bulk soil through six weeks in our study additionally demonstrated that more streptomycetes were associated with the lettuce in the rhizosphere than in surrounding growth substrate. Plants are serving as filters for their associated microbiome. Although Gram-positive bacteria were found as large groups in rhizosphere of many plants, the plant species itself shaped the predominant microbiome compositions. *Bacillus* species were dominant in the rhizosphere of poaceae, sweet-potato, and barley (Normander and Prosser, 2000; Ferrero *et al.*, 2010; Marques *et al.*, 2014). *Arthrobacter* spp. was the most abundant in the rhizosphere of oilseed rape and maize (Gomes *et al.*, 2001; Costa *et al.*, 2006). Actinomycetes spp. were the second most-abundant group in the grass rhizosphere (McCaig *et al.*, 1999). *Streptomyces* species were the dominant communities in the strawberry rhizosphere (Smalla *et al.*, 2001; Costa *et al.*, 2006). The stable lettuce rhizosphere and root colonization by our *Streptomyces* strains indicated their possible long-term interactions. *Streptomyces* could be chemoattracted to the rhizosphere of the growing seedling, where they quickly established a stable interaction with the lettuce.

Different strategies are being used for studying BCAs and PGPB in the rhizosphere. Their localization in roots and seeds rely on microscopic tools exploiting fluorescent markers, which give a fundamental insight into the spatial distribution of the microorganism in the plant rhizosphere and root system (Olivain *et al.*, 2006; Compant *et al.*, 2010; Cardinale, 2014). Application of fluorescent proteins or FISH to study the plant colonization has been widely reported in BCAs and PGPB, including different rhizobacteria, like *Bacillus* and *Pseudomonas* (Buddrus-Schiemann *et al.*, 2010; de-Bashan *et al.*, 2010; Krzyzanowska *et al.*, 2012; Sun *et al.*, 2014). However, particularly for *Streptomyces*, very few studies investigating colonization patterns on plants by using fluorescent proteins in combination with CLSM were addressed. In a previous study, Coombs and Franco (2003) demonstrated that the EGFP-tagged endophytic *Streptomyces* sp. strain EN27 rapidly colonized the wheat embryo, as it was detected in developing seeds as early as 24 h after inoculation. But longer period rhizosphere competence and colonization on roots were not determined. Similarly, Johnson et al. (2008) examined the colonization of several-day-old radish seedling by a pathogenic *Streptomyces* strain using chemical staining. Further monitoring of *Streptomyces* in rhizosphere and radish root tissue was not followed. In our study, we observed the lettuce colonization by EGFP-tagged *S. cyaneus* ZEA17I up to two weeks, which could be the first report examining plant colonization by EGFP-tagged *Streptomyces* in the rhizosphere and root tissue for longer periods than several days. Filamentous growth and spore producing colonization patterns of *Streptomyces* showed their different life cycle with other non-filamentous and non-sporulating rhizobacteria. EGFP-ZEA17I strain rapidly colonized the lettuce seedling root system, and established interactions with lettuce in early stage of lettuce seed germination and root development. This was in agreement with Coombs and Franco's (2003) study that *Streptomyces* rapidly colonize plant tissues when delivered by seed coating method.

In this study, FISH in combination with CLSM was also used as an alternative method to study lettuce colonization by *Streptomyces*, as well as to re-confirm the colonization patterns obtained by the EGFP approach. Both bacteria-universal probe, Cy-3 (EUB338MIX), and *Streptomyces* specific probe, Cy-5 (Strepto), successfully annealed the EGFP-ZEA17I strain. The colonization patterns of the strain examined by FISH-CLSM approach was in accordance with the patterns obtained by CLSM for its fluorescence derived from EGFP as describe above.

Although it is not known if the localization affects its potential for biocontrol and PGP, it has been hypothesized that endophytic bacteria form more stable interactions with plants, rather than rhizospheric or epiphytic bacteria (Ryan *et al.*, 2008; Compant *et al.*, 2010; Malfanova *et al.*, 2011). In this study, both strains were recovered from lettuce inner-root tissue after surface sterilization. However, from the CLSM observations, it was hard to determine the endophytic colonization patterns. Further investigations should be done to examine the endophytic properties of the strains. Other techniques could be applied, e.g. scanning electronic microscopy (SEM), which helped to observe endophytic *Streptomyces* hyphae inside surface sterile tomato root (Sardi *et al.*, 1992). Additionally, quantification by CFU to study colonization dynamics is a widely used method, but bias may exist since *Streptomyces* are filamentous and spore-producing bacteria. Therefore, qPCR could be considered as supplementary method to quantify the population dynamics. The biological activities of the strains used in this thesis work, like their biocontrol of *S. sclerotiorum* causing lettuce basal drop, and growth promotion of different vegetables, are under investigation in other experiments under the same project. Indeed, *S. cyaneus* ZEA17I, whose transformed strain was examined for lettuce colonization, showed PGP activities to different plants, and also showed significant suppression of *S. sclerotiorum in vivo*.

Further experiments to study the colonization of tagged *Streptomyces* in the presence of the *S. sclerotiorum* could be very interesting. On one hand, different factors may affect the ability of rhizobacteria to colonize plant, including phytopathogen infection. For instance, phytopathogens may also influence root exudates and thereby potentially influence the rhizobacteria composition. Biochemical evidence, enhanced exudation of malic acid in the rhizosphere, showed that *Arabidopsis thaliana* Heynh attracted specific rhizosphere colonizers via root exudation after infection with *Pseudomonas syringae* Van Hall (Rudrappa *et al.*, 2008). On the other hand, successful BCA or PGPB have to be highly competitive to successfully colonize the plant rhizosphere, and to build the beneficial interactions with the plants (Compant *et al.*, 2005). In addition, the effects of plant species and soil types on *Streptomyces* rhizospheric and endophytic colonization could be extended research topic for future work. Different plant species produce different root exudates, and thereby shape the preferred rhizosphere colonizers, and lead to specific plant-microbe colonization (Berg and Smalla, 2009). *Actinobacteria* were one of the core groups in *Arabidopsis thaliana* rhizosphere, as well as important composition of the root endophytic microbiota, that was shaped by the soil type (Bulgarelli *et al.*, 2012). The interactions between plant (including its root exudates), microbes, and soil type may finally result in different plant growth performance (Neumann *et al.*, 2014; Schreiter *et al.*, 2014). By using lettuce as a model plant, Schreiter et al. (2014) found that the abundance of root growth-promoting rhizobacteria in three different soil types coincided well with the order of the root length development in soil types. The observed differences in root growth on the different soils may due to the impact of soil type on lettuce root exudates, which shifted the abundance of root growth-promoting rhizobacteria. Connected to biocontrol studies, the complex inter-influences among these factors, plant species, root exudates, soil properties, beneficial rhizobacteria, and the pathogens should be addressed to understand the biocontrol mechanisms of microbe-mediated biocontrol.

In general, this dissertation work applied different approaches to study plant (lettuce) *Streptomyces* colonization. The cultivation method in combination of introduced antibiotics enables to quantify the population dynamics of *Streptomyces* at different soil and lettuce compartments: bulk soil, rhizosphere, and inner-root tissue. Tagging *Streptomyces* with EGFP gene, as well as using FISH probes to stain *Streptomyces* cells, in combination with CLSM provides some notable information about the colonization patterns of lettuce root system by *Streptomyces*. Additionally, the successful transformed strains that maintained biological fitness evaluated in this study that expressing EGFP and apramycin resistance could be used in future studies mentioned above.

Conclusions:

Based on the discussion of the results obtained for each hypothesis of the study, the following conclusions could be made:

1. Transformation of the *Streptomyces* strains with the pIJ8641 constructs harboring the EGFP and apramycin resistance gene was successful.

2. All transformed *Streptomyces* maintained the biological features evaluated, including antagonisms against *Sclerotinia sclerotiorum*, sporulation and mycelium biomass growth, production of secondary metabolites and lytic enzymes.

3. Both *Streptomyces cyaneus* EGFP-ZEA17I, and *Streptomyces* sp. EGFP-SW06W showed relatively stable colonization dynamics in lettuce bulk soil, rhizosphere, and inner-root tissues.

4. Both strains showed significantly higher lettuce rhizosphere and inner-root tissue competences than in bulk soil.

5. The rhizosphere/bulk soil ratio decreased after six weeks for the two strains.

6. In six-week-old plants, *Streptomyces* sp. EGFP-SW06W showed higher competences than *Streptomyces cyaneus* EGFP-ZEA17I both in rhizosphere and inner-root tissue.

7. Based on CLSM observations with EGFP and FISH approaches, *Streptomyces cyaneus* EGFP-ZEA17I rapidly colonized lettuce root systems and started filamentous growth and sporulation within several days after bacterization.

8. *Streptomyces cyaneus* EGFP-ZEA17I colonized lettuce root system up to two weeks, which was in accordance with the quantification of population dynamics.

Acknowledgements

My final personal feeling about doing PhD is an integrated training process of researchers. We joined a research group, forced ourselves for innovation ideas in contribution to a project, collected data for thesis, attended scientific congress and events, build academic relations and collaboration opportunities, wrote the meeting posters, prepare conference oral presentations, and mostly importantly, tried our best to publish the results in peer-reviewed journals to let the colleagues know your work.

We visited different countries and cities by the way of attending scientific meetings. Few chances, we danced and enjoyed beers for nightlife with other scientists. We almost forgot our hobbies, and sometime we may feel a little lonely until found other PhD students are experiencing quite similar things. I am grateful to have such special period in my life. It trained me from a student who always underestimated things to a more peaceful person. It brought me to know science and academia. Academia is a strict, realistic, intelligent, creative, and high level field that searching solutions, basically or practically for all human beings. Different people are demonstrating different talents in science and academia. I am happy to witness my weakness and small advantages to further contribute to science. Thus, doing PhD also helps me to know myself better.

I am extremely grateful to my PhD supervisor, Prof. Paolo Cortesi, world-known plant pathologist for his pioneer work on *Cryphonectria parasitica* vegetative compatibility and hypovirulence for biological control of chestnut blight. It was him to accept me as PhD student in his group, which made me able to experience things above mentioned. I also must thank his patience, encouragement, and guidance to our PhD research. He also gave me a model to be not only a good scientist, also a highly welcomed person in university life, like teaching and administration. Without his spiritual and practical supports, my PhD work could not be done.

I am at the same level grateful to my colleagues in University of Milan, including my co-supervisor Prof. Marco Saracchi, internationally-known for his high cited work in plant associated *Streptomyces*. Special thanks to Dr. Andrea Kunova, the postdoc in our group. It is she taught me basic things in the lab, and guided me for experiments in early stage of my PhD career. Later, her tremendous help and contribution to improve my manuscripts finally lead to paper published. Her talents in organizing things, rigorous attitude of scholarship, and high scientific output give me a model of what a good scientists. I also very impressed about another PhD student, Dr. Maria Bonaldi, now is going to show her talents in the industry. She guided me the culture of Italy, and how makes a person to be loved by everyone around. Her concentration to lab work, and abilities in collecting data, and manage experimental things are highly appreciated. I should learn from her a lot, and Maria, thanks, very much-also for your generous cooperation and team working attitude. I also want to thank Dr. Cristina Pizzatti for her contribution in helping my experiments, especially assistance in statistical analysis. Standing aloof from the world is the unique property and attitude. She never tries to be angry with you, even when you messed things up. I think her boyfriend is one of the happiest men in the world.

I also want to thank all the stuffs of the CBEA PhD School. It should be one of the best organized doctorate schools in Italy. First, the PhD school organized annual workshop totally as a conference. I know other schools also did, but our school arranged in days, and every PhD students got a chance to make oral presentation in the workshop. I cannot forget one year, our workshop was held in Sala Napoleonica, one of the most beautiful churches in Italy. Secondly, our PhD school provided abundant travel grants for students to attending national and international conferences. Finally, the research and education components of our school are impressive. We almost cover all the domains in agricultural related topics. Theory and lab courses, as well as seminars frequently the international speakers presented improved our knowledge and skills.

I also want to thank the collaborators in this PhD work. Prof. Flavia Marinelli (University of Insubria, Italy) and Prof. Mervyn Bibb (John Innes Centre, UK) kindly provided plasmids and reference strains. Special thanks to Prof. Gabriele Berg (Graz University of Technology, Austria) for holding me three months in her lab, to cooperate the CLSM microscopy part of the work. It is a very well organized and advance lab in microbial ecology. The same level thanks to my collaborator Dr. Armin Erlacher, for his kind assistance and guidance in CLSM. Dr. Tomislav Cernava and Mr. Tobija Glawogger are also acknowledged for technical discussion and assistance during experiments.

Finally, I must thank my families, especially my wife Qian. We had a baby born while I was starting PhD. It was not easy, but we survived. I am very grateful that she took the most responsibilities in looking after our son, Siqi Chen, in last three years. To my wife, I want to say, I will give you back. I should also thank my son, Siqi, you are quite naught, but very healthy, which make me could concentrate on PhD work. Sorry to you, that makes you far away grandma and relatives. Many thanks to my Mother, Miss Wu for financial supports to raise Siqi up. Finally, I want to express my love, miss, and sorry to my father, Mr. Shaoxue Chen. He passed away by accident when I was doing master degree in University of Helsinki, Finland. One week before the accident, he told me he had a wish that I could go further for studies, like doing a PhD. Also in that strange week, he wrote a motto on paper and hanged on the wall: 敬学如敬人，百善孝为先 （Respect research as respecting human, the best quality in the world is filial piety）. To be honest, until now, I don’t understand what the connection of the two sentences is. However, I will try my best to follow them individually- doing honest research and treating families well.

So, as a conclusion, this PhD thesis is dedicated to all the people whom ever helped me in my career, also my father.

References

Acinas, S.G., Sarma-Rupavtarm, R., Klepac-Ceraj, V., Polz, M.F., 2005. PCR-induced sequence artifacts and bias: Insights from comparison of two 16S rRNA clone libraries constructed from the same sample. Appl Environ Microb 71, 8966-8969.

Alabouvette, C., Olivain, C., Steinberg, C., 2006. Biological control of plant diseases: the European situation. Eur J Plant Pathol 114, 329-341.

Altieri, M.A., 1991. Classical Biological-Control and Social Equity. B Entomol Res 81, 365-369.

Alvarez, F., Castro, M., Principe, A., Borioli, G., Fischer, S., Mori, G., Jofre, E., 2012. The plant-associated Bacillus amyloliquefaciens strains MEP218 and ARP23 capable of producing the cyclic lipopeptides iturin or surfactin and fengycin are effective in biocontrol of sclerotinia stem rot disease. J Appl Microbiol 112, 159-174.

Amann, R., Fuchs, B.M., 2008. Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. Nat Rev Microbiol 6, 339-348.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16s Ribosomal-Rna-Targeted Oligonucleotide Probes with Flow-Cytometry for Analyzing Mixed Microbial-Populations. Appl Environ Microb 56, 1919-1925.

Atallah, Z.K., Hayes, R.J., Subbarao, K.V., 2011. Fifteen Years of Verticillium Wilt of Lettuce in America's Salad Bowl: A Tale of Immigration, Subjugation, and Abatement. Plant Dis 95, 784-792.

Audenaert, K., Pattery, T., Cornelis, P., Hofte, M., 2002. Induction of systemic resistance to Botrytis cinerea in tomato by Pseudomonas aeruginosa 7NSK2: Role of salicylic acid, pyochelin, and pyocyanin. Mol Plant Microbe In 15, 1147-1156.

Bano, N., Musarrat, J., 2003. Characterization of a new Pseudomonas aeruginosa strain NJ-15 as a potential biocontrol agent. Curr Microbiol 46, 324-328.

Barbour, E.K., Husseini, S.A., Farran, M.T., Itani, D.A., Houalla, R.H., Hamadeh, S.K., 2002. Soil solarization: A sustainable agriculture approach to reduce microorganisms in chicken manure-treated soil. J Sustain Agr 19, 95-104.

Barratt, B.I.P., Howarth, F.G., Withers, T.M., Kean, J.M., Ridley, G.S., 2010. Progress in risk assessment for classical biological control. Biol Control 52, 245-254.
Bentley, S.D., Chater, K.F., Cerdeno-Tarraga, A.M., Challis, G.L., Thomson, N.R., James, K.D., Harris, D.E., Quail, M.A., Kieser, H., Harper, D., Bateman, A., Brown, S., Chandra, G., Chen, C.W., Collins, M., Cronin, A., Fraser, A., Goble, A., Hidalgo, J., Hornsby, T., Howarth, S., Huang, C.H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O'Neil, S., Rabbinowitsch, E., Rajandream, M.A., Rutherford, K., Rutter, S., Seeger, K., Saunders, D., Sharp, S., Squares, R., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B.G., Parkhill, J., Hopwood, D.A., 2002. Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2). Nature 417, 141-147.

Berg, G., Smalla, K., 2009. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. Fems Microbiol Ecol 68, 1-13.

Bhattacharyya, P.N., Jha, D.K., 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J Microbiol Biotechnol 28, 1327-1350.

Bloemberg, G.V., Lugtenberg, B.J.J., 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr Opin Plant Biol 4, 343-350.

Blot, M., Hauer, B., Monnet, G., 1994. The Tn5 Bleomycin Resistance Gene Confers Improved Survival and Growth Advantage on Escherichia-Coli. Mol Gen Genet 242, 595-601.

Bonaldi, M., Chen, X.Y.L., Kunova, A., Pizzatti, C., Saracchi, M., Cortesi, P., 2015. Colonization of lettuce rhizosphere and roots by tagged Streptomyces. Frontiers in Microbiology 6.

Bonaldi, M., Kunova, A., Saracchi, M., Sardi, P., Cortesi, P., 2014. Streptomycetes as Biological Control Agents against Basal Drop. Viii International Symposium on Chemical and Non-Chemical Soil and Substrate Disinfestation 1044, 313-318.

Bonanomi, G., Chiurazzi, M., Caporaso, S., Del Sorbo, G., Moschetti, G., Felice, S., 2008. Soil solarization with biodegradable materials and its impact on soil microbial communities. Soil Biol Biochem 40, 1989-1998.

Bouchez, T., Patureau, D., Dabert, P., Juretschko, S., Dore, J., Delgenes, P., Moletta, R., Wagner, M., 2000. Ecological study of a bioaugmentation failure. Environ Microbiol 2, 179-190.

Brader, G., Compant, S., Mitter, B., Trognitz, F., Sessitsch, A., 2014. Metabolic potential of endophytic bacteria. Curr Opin Biotech 27, 30-37.

Bric, J.M., Bostock, R.M., Silverstone, S.E., 1991. Rapid in situ assay for indoleacetic Acid production by bacteria immobilized on a nitrocellulose membrane. Appl Environ Microbiol 57, 535-538.

Britigan, B.E., Roeder, T.L., Rasmussen, G.T., Shasby, D.M., Mccormick, M.L., Cox, C.D., 1992. Interaction of the Pseudomonas-Aeruginosa Secretory Products Pyocyanin and Pyochelin Generates Hydroxyl Radical and Causes Synergistic Damage to Endothelial-Cells - Implications for Pseudomonas-Associated Tissue-Injury. J Clin Invest 90, 2187-2196.

Broadley, M.R., Escobar-Gutierrez, A.J., Burns, A., Burns, I.G., 2000. What are the effects of nitrogen deficiency on growth components of lettuce? New Phytol 147, 519-526.

Buddrus-Schiemann, K., Schmid, M., Schreiner, K., Welzl, G., Hartmann, A., 2010. Root Colonization by Pseudomonas sp DSMZ 13134 and Impact on the Indigenous Rhizosphere Bacterial Community of Barley. Microb Ecol 60, 381-393.

Budge, S.P., Mcquilken, M.P., Fenlon, J.S., Whipps, J.M., 1995. Use of Coniothyrium-Minitans and Gliocladium-Virens for Biological-Control of Sclerotinia-Sclerotiorum in Glasshouse Lettuce. Biol Control 5, 513-522.

Budge, S.P., Whipps, J.M., 1991. Glasshouse Trials of Coniothyrium-Minitans and Trichoderma Species for the Biological-Control of Sclerotinia-Sclerotiorum in Celery and Lettuce. Plant Pathol 40, 59-66.

Bulgarelli, D., Rott, M., Schlaeppi, K., van Themaat, E.V.L., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., Schmelzer, E., Peplies, J., Gloeckner, F.O., Amann, R., Eickhorst, T., Schulze-Lefert, P., 2012. Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. Nature 488, 91-95.

Cao, L., Qiu, Z., You, J., Tan, H., Zhou, S., 2004. Isolation and characterization of endophytic Streptomyces strains from surface-sterilized tomato (Lycopersicon esculentum) roots. Letters in Applied Microbiology 39, 425-430.

Cardinale, M., 2014. Scanning a microhabitat: plant-microbe interactions revealed by confocal laser microscopy. Frontiers in Microbiology 5.

Cardinale, M., de Castro, J.V., Muller, H., Berg, G., Grube, M., 2008. In situ analysis of the bacterial community associated with the reindeer lichen Cladonia arbuscula reveals predominance of Alphaproteobacteria. Fems Microbiol Ecol 66, 63-71.

Chater K F, B.M.J., 1996. Regulation of bacterial antibiotic production. In: Kleinkauf H, v.D.H. (Ed.), Products of secondary metabolism. Biotechnology VCH, Weinheim, Germany, pp. 57-105.

Chellemi, D.O., Olson, S.M., Mitchell, D.J., Secker, I., McSorley, R., 1997. Adaptation of soil solarization to the integrated management of soilborne pests of tomato under humid conditions. Phytopathology 87, 250-258.

Chen, C., Feng, W.W., Qin, S., Zhao, X.Q., 2015. Streptomyces xiaopingdaonensis sp nov., a novel marine actinomycete isolated from the sediment of Xiaopingdao in Dalian, China. Anton Leeuw Int J G 107, 511-518.

Chitrampalam, P., Turini, T.A., Pryor, B.M., 2008. Effect of biocontrol and chemical strategies against lettuce drop caused by Sclerotinia sclerotiorum and S-minor in desert agroecosystems. Phytopathology 98, S38-S38.

Compant, S., Clement, C., Sessitsch, A., 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biol Biochem 42, 669-678.

Compant, S., Duffy, B., Nowak, J., Clement, C., Barka, E.A., 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: Principles, mechanisms of action, and future prospects. Appl Environ Microb 71, 4951-4959.

Compant, S., Kaplan, H., Sessitsch, A., Nowak, J., Barka, E.A., Clement, C., 2008. Endophytic colonization of Vitis vinifera L. by Burkholderia phytofirmans strain PsJN: from the rhizosphere to inflorescence tissues. Fems Microbiol Ecol 63, 84-93.

Cook, S.M., Khan, Z.R., Pickett, J.A., 2007. The use of push-pull strategies in integrated pest management. Annu Rev Entomol 52, 375-400.

Coombs, J.T., Franco, C.M.M., 2003. Visualization of an endophytic Streptomyces species in wheat seed. Appl Environ Microb 69, 4260-4262.

Costa, R., Gotz, M., Mrotzek, N., Lottmann, J., Berg, G., Smalla, K., 2006. Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. Fems Microbiol Ecol 56, 236-249.

Culman, S.W., Duxbury, J.M., Lauren, J.G., Thies, J.E., 2006. Microbial community response to soil solarization in Nepal's rice-wheat cropping system. Soil Biol Biochem 38, 3359-3371.

Dahiya, N., Tewari, R., Hoondal, G.S., 2006. Biotechnological aspects of chitinolytic enzymes: a review. Appl Microbiol Biot 71, 773-782.

Daims, H., Bruhl, A., Amann, R., Schleifer, K.H., Wagner, M., 1999. The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. Syst Appl Microbiol 22, 434-444.
de-Bashan, L.E., Hernandez, J.P., Bashan, Y., Maier, R.M., 2010. Bacillus pumilus ES4: Candidate plant growth-promoting bacterium to enhance establishment of plants in mine tailings. Environ Exp Bot 69, 343-352.

de Ponti, T., Rijk, B., van Ittersum, M.K., 2012. The crop yield gap between organic and conventional agriculture. Agr Syst 108, 1-9.

Demain, A.L., 2009. Antibiotics: Natural Products Essential to Human Health. Med Res Rev 29, 821-842.

ECPA, 2009. ECPA position on the future revision of Regulations 1107/2009 and 396/2005. , 2009. . European Commission, Brussels, p. PP/12/EJ/22085.

Emmert, E.A.B., Handelsman, J., 1999. Biocontrol of plant disease: a (Gram-) positive perspective. Fems Microbiol Lett 171, 1-9.

Erlacher, A., Cardinale, M., Grube, M., Berg, G., 2015. Biotic Stress Shifted Structure and Abundance of Enterobacteriaceae in the Lettuce Microbiome. Plos One 10.

Errampalli, D., Leung, K., Cassidy, M.B., Kostrzynska, M., Blears, M., Lee, H., Trevors, J.T., 1999. Applications of the green fluorescent protein as a molecular marker in environmental microorganisms. J Microbiol Meth 35, 187-199.

FAO, 2014. FAOSTAT. FAO, Rome, Italy.

Faria, J.C., Jelihovschi, E. G., and Allaman, I. B. , 2013. Conventional Tukey Test. Ilheus: Test.UESC.

Fernando, W.G.D., Nakkeeran, S., Zhang, Y., Savchuk, S., 2007. Biological control of Sclerotinia sclerotiorum (Lib.) de Bary by Pseudomonas and Bacillus species on canola petals. Crop Prot 26, 100-107.

Ferrero, M.A., Menoyo, E., Lugo, M.A., Negritto, M.A., Farias, M.E., Anton, A.M., Sineriz, F., 2010. Molecular characterization and in situ detection of bacterial communities associated with rhizosphere soil of high altitude native Poaceae from the Andean Puna region. J Arid Environ 74, 1177-1185.

Fravel, D.R., 1988. Role of Antibiosis in the Biocontrol of Plant-Diseases. Annu Rev Phytopathol 26, 75-91.

Gamalero, E., Lingua, G., Berta, G., Lemanceau, P., 2003. Methods for studying root colonization by introduced beneficial bacteria. Agronomie 23, 407-418.

Gamalero, E., Lingua, G., Tombolini, R., Avidano, L., Pivato, B., Berta, G., 2005. Colonization of tomato root seedling by Pseudomonas fluorescens 92rkG5: Spatio-temporal dynamics, localization, organization, viability, and culturability. Microb Ecol 50, 289-297.

Garibaldi, A., Gilardi, G., Gullino, M.L., 2002. First Report of Fusarium oxysporum on Lettuce in Europe. Plant Dis 86, 1052-1052.

Garibaldi, A., Gilardi, G., Gullino, M.L., 2004a. Seed transmission of *Fusarium oxysporum* f.sp. *lactucae*. Phytoparasitica 32, 61-65.

Garibaldi, A., Gilardi, G., Gullino, M.L., 2004b. Varietal resistance of lettuce to Fusarium oxysporum f. sp lactucae. Crop Prot 23, 845-851.

Garibaldi, A., Gilardi, G., Gullino, M.L., 2005. First report of root rot incited by *Thielaviopsis basicola* on lamb's lettuce (*Valerianella olitoria*) in Europe. Plant Disease 89, 205-205.

Garibaldi, A., Gilardi, G., Gullino, M.L., 2014. Critical Aspects in Disease Management as a Consequence of the Evolution of Soil-Borne Pathogens. Viii International Symposium on Chemical and Non-Chemical Soil and Substrate Disinfestation 1044, 43-50.

Getha, K., Vikineswary, S., Wong, W.H., Seki, T., Ward, A., Goodfellow, M., 2005. Evaluation of Streptomyces sp. strain g10 for suppression of Fusarium wilt and rhizosphere colonization in pot-grown banana plantlets. J IND MICROBIOL BIOTECHNOL 32, 24-32.

Glandorf, D.C.M., Brand, I., Bakker, P.A.H.M., Schippers, B., 1992. Stability of Rifampicin Resistance as a Marker for Root Colonization Studies of Pseudomonas-Putida in the Field. Plant Soil 147, 135-142.

Glick, B., 2015. Biocontrol Mechanisms. Beneficial Plant-Bacterial Interactions. Springer International Publishing, pp. 123-157.

Gomes, N.C.M., Heuer, H., Schonfeld, J., Costa, R., Mendonca-Hagler, L., Smalla, K., 2001. Bacterial diversity of the rhizosphere of maize (Zea mays) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant Soil 232, 167-180.

Grantcharova, N., Lustig, U., Flardh, K., 2005. Dynamics of FtsZ assembly during sporulation in Streptomyces coelicolor A3(2). J Bacteriol 187, 3227-3237.

Gupta, R., Saxena, R.K., Chaturvedi, P., Virdi, J.S., 1995. Chitinase Production by Streptomyces Viridificans - Its Potential in Fungal Cell-Wall Lysis. J Appl Bacteriol 78, 378-383.

Haas, D., Defago, G., 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 3, 307-319.

Haas, J., Park, E.C., Seed, B., 1996. Codon usage limitation in the expression of HIV-1 envelope glycoprotein. Curr Biol 6, 315-324.

Hamdali, H., Hafidi, M., Virolle, M.J., Ouhdouch, Y., 2008. Growth promotion and protection against damping-off of wheat by two rock phosphate solubilizing actinomycetes in a P-deficient soil under greenhouse conditions. Appl Soil Ecol 40, 510-517.

Hanahan, D., 1983. Studies on transformation of Escherichia coli with plasmids. J Mol Biol 166, 557-580.

Handelsman, J., Stabb, E.V., 1996. Biocontrol of soilborne plant pathogens. Plant Cell 8, 1855-1869.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M., 2004. Trichoderma species - Opportunistic, avirulent plant symbionts. Nat Rev Microbiol 2, 43-56.

Hartmann, A., Schmid, M., van Tuinen, D., Berg, G., 2009. Plant-driven selection of microbes. Plant Soil 321, 235-257.

Haseloff, J., Siemering, K.R., 2005. The Uses of Green Fluorescent Protein in Plants. Green Fluorescent Protein. John Wiley & Sons, Inc., pp. 259-284.

Hiltner, 1904. Über neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie unter besonderer Berücksichtigung der Gründüngung und Brache. Arb. Dtsch. Landwirtsch. Ges 98.

Hofmann, A., Fischer, D., Hartmann, A., Schmid, M., 2014. Colonization of plants by human pathogenic bacteria in the course of organic vegetable production. Frontiers in Microbiology 5.

Hopwood, D.A., 1988. The Leeuwenhoek Lecture, 1987 - Towards an Understanding of Gene Switching in Streptomyces, the Basis of Sporulation and Antibiotic Production. Proc R Soc Ser B-Bio 235, 121-+.

Hossard, L., Philibert, A., Bertrand, M., Colnenne-David, C., Debaeke, P., Munier-Jolain, N., Jeuffroy, M.H., Richard, G., Makowski, D., 2014. Effects of halving pesticide use on wheat production. Sci Rep-Uk 4.

Howell, C.R., 2003. Mechanisms employed by Trichoderma species in the biological control of plant diseases: The history and evolution of current concepts. Plant Dis 87, 4-10.

Huang, H.C., Bremer, E., Hynes, R.K., Erickson, R.S., 2000. Foliar application of fungal biocontrol agents for the control of white mold of dry bean caused by Sclerotinia sclerotiorum. Biol Control 18, 270-276.

Hubbard, J.C., Gerik, J.S., 1993. A New Wilt Disease of Lettuce Incited by Fusarium-Oxysporum F Sp Lactucum Forma Specialis Nov. Plant Dis 77, 750-754.

Inbar, J., Menendez, A., Chet, I., 1996. Hyphal interaction between Trichoderma harzianum and Sclerotinia sclerotiorum and its role in biological control. Soil Biol Biochem 28, 757-763.

Jacobsen, B.J., Zidack, N.K., Larson, B.J., 2004. The role of Bacillus-based biological control agents in integrated pest management systems: Plant diseases. Phytopathology 94, 1272-1275.

Jansson, J.K., 1995. Tracking Genetically-Engineered Microorganisms in Nature. Curr Opin Biotech 6, 275-283.

Jog, R., Pandya, M., Nareshkumar, G., Rajkumar, S., 2014. Mechanism of phosphate solubilization and antifungal activity of Streptomyces spp. isolated from wheat roots and rhizosphere and their application in improving plant growth. Microbiol-Sgm 160, 778-788.

Johansson, E., Reponen, T., Meller, J., Vesper, S., Yadav, J., 2014. Association of Streptomyces community composition determined by PCR-denaturing gradient gel electrophoresis with indoor mold status. Environ Monit Assess 186, 8773-8783.

Johnson, E.G., Sparks, J.P., Dzikovski, B., Crane, B.R., Gibson, D.M., Loria, R., 2008. Plant-pathogenic Streptomyces species produce nitric oxide synthase-derived nitric oxide in response to host signals. Chem Biol 15, 43-50.

Juretschko, S., Loy, A., Lehner, A., Wagner, M., 2002. The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. Syst Appl Microbiol 25, 84-99.

Kadam, S.S., Salunkhe, D.D.K., 1988. Handbook of Vegetable Science and Technology: Production, Composition, Storage, and Processing. pp. 495–. Retrieved 21 May 2013. Taylor & Francis.

Katan, J., Grinstein, A., Greenberger, A., Yarden, O., Devay, J.E., 1987. The 1st Decade (1976-1986) of Soil Solarization (Solar Heating) - a Chronological Bibliography. Phytoparasitica 15, 229-255.

Keel, C., Voisard, C., Berling, C.H., Kahr, G., Defago, G., 1989. Iron Sufficiency, a Prerequisite for the Suppression of Tobacco Black Root-Rot by Pseudomonas-Fluorescens Strain Chao under Gnotobiotic Conditions. Phytopathology 79, 584-589.
Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., Hopwood, D.A., 2000. Practical *Streptomyces* Genetics. The John Innes Foundation, Norwish, UK.

Kingston, W., 2004. Streptomycin, Schatz v. Waksman, and the balance of credit for discovery. J Hist Med All Sci 59, 441-462.

Kliot, A., Kontsedalov, S., Lebedev, G., Brumin, M., Cathrin, P.B., Marubayashi, J.M., Skaljac, M., Belausov, E., Czosnek, H., Ghanim, M., 2014. Fluorescence in situ Hybridizations (FISH) for the Localization of Viruses and Endosymbiotic Bacteria in Plant and Insect Tissues. Jove-J Vis Exp.

Kloepper, J., Leong, J., Teintze, M., Schroth, M., 1980. Pseudomonas siderophores: A mechanism explaining disease-suppressive soils. Curr Microbiol 4, 317-320.

Kogan, M., 1998. Integrated pest management: Historical perspectives and contemporary developments. Annu Rev Entomol 43, 243-270.

Kohler, H.R., Triebskorn, R., 2013. Wildlife Ecotoxicology of Pesticides: Can We Track Effects to the Population Level and Beyond? Science 341, 759-765.

Kremers, G.J., Gilbert, S.G., Cranfill, P.J., Davidson, M.W., Piston, D.W., 2011. Fluorescent proteins at a glance. J Cell Sci 124, 157-160.

Krzyzanowska, D., Obuchowski, M., Bikowski, M., Rychlowski, M., Jafra, S., 2012. Colonization of Potato Rhizosphere by GFP-Tagged Bacillus subtilis MB73/2, Pseudomonas sp P482 and Ochrobactrum sp A44 Shown on Large Sections of Roots Using Enrichment Sample Preparation and Confocal Laser Scanning Microscopy. Sensors-Basel 12, 17608-17619.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S.C., Bron, S., Brouillet, S., Bruschi, C.V., Caldwell, B., Capuano, V., Carter, N.M., Choi, S.K., Codani, J.J., Connerton, I.F., Cummings,

N.J., Daniel, R.A., Denizot, F., Devine, K.M., Dusterhoft, A., Ehrlich, S.D., Emmerson, P.T., Entian, K.D., Errington, J., Fabret, C., Ferrari, E., Foulger, D., Fritz, C., Fujita, M., Fujita, Y., Fuma, S., Galizzi, A., Galleron, N., Ghim, S.Y., Glaser, P., Goffeau, A., Golightly, E.J., Grandi, G., Guiseppi, G., Guy, B.J., Haga, K., Haiech, J., Harwood, C.R., Henaut, A., Hilbert, H., Holsappel, S., Hosono, S., Hullo, M.F., Itaya, M., Jones, L., Joris, B., Karamata, D., Kasahara, Y., KlaerrBlanchard, M., Klein, C., Kobayashi, Y., Koetter, P., Koningstein, G., Krogh, S., Kumano, M., Kurita, K., Lapidus, A., Lardinois, S., Lauber, J., Lazarevic, V., Lee, S.M., Levine, A., Liu, H., Masuda, S., Mauel, C., Medigue, C., Medina, N., Mellado, R.P., Mizuno, M., Moestl, D., Nakai, S., Noback, M., Noone, D., OReilly, M., Ogawa, K., Ogiwara, A., Oudega, B., Park, S.H., Parro, V., Pohl, T.M., Portetelle, D., Porwollik, S., Prescott, A.M., Presecan, E., Pujic, P., Purnelle, B., Rapoport, G., Rey, M., Reynolds, S., Rieger, M., Rivolta, C., Rocha, E., Roche, B., Rose, M., Sadaie, Y., Sato, T., Scanlan, E., Schleich, S., Schroeter, R., Scoffone, F., Sekiguchi, J., Sekowska, A., Seror, S.J., Serror, P., Shin, B.S., Soldo, B., Sorokin, A., Tacconi, E., Takagi, T., Takahashi, H., Takemaru, K., Takeuchi, M., Tamakoshi, A., Tanaka, T., Terpstra, P., Tognoni, A., Tosato, V., Uchiyama, S., Vandenbol, M., Vannier, F., Vassarotti, A., Viari, A., Wambutt, R., Wedler, E., Wedler, H., Weitzenegger, T., Winters, P., Wipat, A., Yamamoto, H., Yamane, K., Yasumoto, K., Yata, K., Yoshida, K., Yoshikawa, H.F., Zumstein, E., Yoshikawa, H., Danchin, A., 1997. The complete genome sequence of the Gram-positive bacterium Bacillus subtilis. Nature 390, 249-256.

Lamberth, C., Jeanmart, S., Luksch, T., Plant, A., 2013. Current Challenges and Trends in the Discovery of Agrochemicals. Science 341, 742-746.

Lemanceau, P., Bakker, P.A.H.M., Dekogel, W.J., Alabouvette, C., Schippers, B., 1992. Effect of Pseudobactin-358 Production by Pseudomonas-Putida Wcs358 on Suppression of Fusarium-Wilt of Carnations by Nonpathogenic Fusarium-Oxysporum Fo47. Appl Environ Microb 58, 2978-2982.

Li, G.Q., Wang, D.B., Jiang, D.H., Huang, H.C., Laroche, A., 2000. First report of Sclerotinia nivalis on lettuce in central China. Mycol Res 104, 232-237.

Liu, X., Yin, Y.N., Yan, L.Y., Michailides, T.J., Ma, Z.H., 2009. Sensitivity to iprodione and boscalid of Sclerotinia sclerotiorum isolates collected from rapeseed in China. Pestic Biochem Phys 95, 106-112.

Llorach, R., Martinez-Sanchez, A., Tomas-Barberan, F.A., Gil, M.I., Ferreres, F., 2008. Characterisation of polyphenols and antioxidant properties of five lettuce varieties and escarole. Food Chem 108, 1028-1038.

Loper, J.E., Buyer, J.S., 1991. Siderophores in Microbial Interactions on Plant-Surfaces. Mol Plant Microbe In 4, 5-13.

Lubeck, M., Knudsen, I.M.B., Jensen, B., Thrane, U., Janvier, C., Jensen, D.F., 2002. GUS and GFP transformation of the biocontrol strain Clonostachys rosea IK726 and the use of these marker genes in ecological studies. Mycol Res 106, 815-826.

Lucy, M., Reed, E., Glick, B.R., 2004. Applications of free living plant growth-promoting rhizobacteria. Anton Leeuw Int J G 86, 1-25.

Lugtenberg, B., Kamilova, F., 2009. Plant-growth-promoting rhizobacteria. Annual review of microbiology 63, 541-556.

Mahadevan, B., Crawford, D.L., 1997. Properties of the chitinase of the antifungal biocontrol agent Streptomyces lydicus WYEC108. Enzyme Microb Tech 20, 489-493.
Malfanova, N., Kamilova, F., Validov, S., Shcherbakov, A., Chebotar, V., Tikhonovich, I., Lugtenberg, B., 2011. Characterization of Bacillus subtilis HC8, a novel plant-beneficial endophytic strain from giant hogweed. Microb Biotechnol 4, 523-532.

Manteca, A., Sanchez, J., 2009. Streptomyces Development in Colonies and Soils. Appl Environ Microb 75, 2920-2924.

Marahatta, S.P., Wang, K.H., Sipes, B.S., Hooks, C.R.R., 2012. Effects of the Integration of Sunn Hemp and Soil Solarization on Plant-Parasitic and Free-Living Nematodes. J Nematol 44, 72-79.

Marques, J.M., da Silva, T.F., Vollu, R.E., Blank, A.F., Ding, G.C., Seldin, L., Smalla, K., 2014. Plant age and genotype affect the bacterial community composition in the tuber rhizosphere of field-grown sweet potato plants. Fems Microbiol Ecol 88, 424-435.

Martinez, J.L., 2008. Antibiotics and antibiotic resistance genes in natural environments. Science 321, 365-367.

Matheron, M.E., Porchas, M., 2008. Effectiveness of the biopesticides Actinovate and Kaligreen within a management program for powdery mildew on cantaloupe. Phytopathology 98, S99-S100.

Matuo, T., Motohashi, S., 1976. On Fusarium oxysporum f. sp. lactucae n. f. causing root rot of lettuce. Trans. Mycol. Soc. Jpn. 8, 13-15.

McCaig, A.E., Glover, L.A., Prosser, J.I., 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl Environ Microb 65, 1721-1730.

Mirleau, P., Delorme, S., Philippot, L., Meyer, J.M., Mazurier, S., Lemanceau, P., 2000. Fitness in soil and rhizosphere of Pseudomonas fluorescens C7R12 compared with a C7R12 mutant affected in pyoverdine synthesis and uptake. Fems Microbiol Ecol 34, 35-44.

Mirleau, P., Philippot, L., Corberand, T., Lemanceau, P., 2001. Involvement of nitrate reductase and pyoverdine in competitiveness of Pseudomonas fluorescens strain C7R12 in soil. Appl Environ Microb 67, 2627-2635.

Mou, B., 2008. Lettuce. In: Prohens, J., Nuez, F. (Eds.), Vegetables I. Springer New York, pp. 75-116.

Nautiyal, C.S., 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. Fems Microbiol Lett 170, 265-270.

Nelson, L.M., 2004. Plant Growth Promoting Rhizobacteria (PGPR): Prospects for New Inoculants. Crop Management 3.

Nemec, S., Datnoff, L.E., Strandberg, J., 1996. Efficacy of biocontrol agents in planting mixes to colonize plant roots and control root diseases of vegetables and citrus. Crop Prot 15, 735-742.

Neumann, G., Bott, S., Ohler, M.A., Mock, H.P., Lippmann, R., Grosch, R., Smalla, K., 2014. Root exudation and root development of lettuce (Lactuca sativa L. cv. Tizian) as affected by different soils. Frontiers in Microbiology 5.

Nielsen, T.H., Sorensen, D., Tobiasen, C., Andersen, J.B., Christophersen, C., Givskov, M., Sorensen, J., 2002. Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent Pseudomonas spp. from the sugar beet rhizosphere. Appl Environ Microb 68, 3416-3423.

Nigro, F., Sialer, M.M.F., Gallitelli, D., 1999. Transformation of Metschnikowia pulcherrima 320, biocontrol agent of storage rot, with the green fluorescent protein gene. J Plant Pathol 81, 205-208.

Normander, B., Prosser, J.I., 2000. Bacterial origin and community composition in the barley phytosphere as a function of habitat and presowing conditions. Appl Environ Microb 66, 4372-4377.

Olivain, C., Humbert, C., Nahalkova, J., Fatehi, J., L'Haridon, F., Alabouvette, C., 2006. Colonization of tomato root by pathogenic and nonpathogenic Fusarium oxysporum strains inoculated together and separately into the soil. Appl Environ Microb 72, 1523-1531.

Pal, K., Gardener, B., 2006. Biological Control of Plant Pathogens. The Plant Health Instructor. APS.

Paulitz, T.C., Belanger, R.R., 2001. Biological control in greenhouse systems. Annu Rev Phytopathol 39, 103-133.

Perez-Miranda, S., Cabirol, N., George-Tellez, R., Zamudio-Rivera, L.S., Fernandez, F.J., 2007. O-CAS, a fast and universal method for siderophore detection. J Microbiol Meth 70, 127-131.

Phillips, A.J.L., 1990. The Effects of Soil Solarization on Sclerotial Populations of Sclerotinia-Sclerotiorum. Plant Pathol 39, 38-43.

Phoebe, C.H., Combie, J., Albert, F.G., Van Tran, K., Cabrera, J., Correira, H.J., Guo, Y.H., Lindermuth, J., Rauert, N., Galbraith, W., Selitrennikoff, C.P., 2001. Extremophilic organisms as an unexplored source of antifungal compounds. J Antibiot 54, 56-65.

Pope, M.K., Green, B.D., Westpheling, J., 1996. The bld mutants of Streptomyces coelicolor are defective in the regulation of carbon utilization, morphogenesis and cell-cell signalling. Mol Microbiol 19, 747-756.

Prieto, P., Schiliro, E., Maldonado-Gonzalez, M.M., Valderrama, R., Barroso-Albarracin, J.B., Mercado-Blanco, J., 2011. Root Hairs Play a Key Role in the Endophytic Colonization of Olive Roots by Pseudomonas spp. with Biocontrol Activity. Microb Ecol 62, 435-445.

R Core Team, 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2012. ISBN 3-900051-07-0.
Raaijmakers, J.M., Vlami, M., de Souza, J.T., 2002. Antibiotic production by bacterial biocontrol agents. Anton Leeuw Int J G 81, 537-547.

Rashad, F.M., Fathy, H.M., El-Zayat, A.S., Elghonaimy, A.M., 2015. Isolation and characterization of multifunctional Streptomyces species with antimicrobial, nematicidal and phytohormone activities from marine environments in Egypt. Microbiol Res 175, 34-47.

Rudrappa, T., Czymmek, K.J., Pare, P.W., Bais, H.P., 2008. Root-Secreted Malic Acid Recruits Beneficial Soil Bacteria. Plant Physiol 148, 1547-1556.

Ryan, R.P., Germaine, K., Franks, A., Ryan, D.J., Dowling, D.N., 2008. Bacterial endophytes: recent developments and applications. Fems Microbiol Lett 278, 1-9.

Sabaratnam, S., Traquair, J.A., 2002. Formulation of a Streptomyces Biocontrol Agent for the Suppression of Rhizoctonia Damping-off in Tomato Transplants. Biol Control 23, 245-253.

Saima, Kuddus, M., Roohi, Ahmad, I.Z., 2013. Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinase. Journal of Genetic Engineering and Biotechnology 11, 39-46.

Sardi, P., Saracchi, M., Quaroni, S., Petrolini, B., Borgonovi, G.E., Merli, S., 1992. Isolation of Endophytic Streptomyces Strains from Surface-Sterilized Roots. Appl Environ Microb 58, 2691-2693.

Schmid, M., Twachtmann, U., Klein, M., Strous, M., Juretschko, S., Jetten, M., Metzger, J.W., Schleifer, K.H., Wagner, M., 2000. Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst Appl Microbiol 23, 93-106.

Schottel, J.L., Shimizu, K., Kinkel, L.L., 2001. Relationships of in Vitro Pathogen Inhibition and Soil Colonization to Potato Scab Biocontrol by Antagonistic Streptomyces spp. Biol Control 20, 102-112.

Schreiter, S., Ding, G.C., Heuer, H., Neumann, G., Sandmann, M., Grosch, R., Kropf, S., Smalla, K., 2014. Effect of the soil type on the microbiome in the rhizosphere of field-grown lettuce. Frontiers in Microbiology 5.

Schwyn, B., Neilands, J.B., 1987. Universal Chemical-Assay for the Detection and Determination of Siderophores. Anal Biochem 160, 47-56.

Simon, R., Priefer, U., Puhler, A., 1983. A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. Nat Biotech 1, 784-791.

Sipos, R., Székely, A., Révész, S., Márialigeti, K., 2010. Addressing PCR Biases in Environmental Microbiology Studies. pp. 37-58.

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H., Berg, G., 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. Appl Environ Microb 67, 4742-4751.

Smith, H.S., 1919. On some phases of insect control by the biological method. Journal of Economic Entomology 288-292.

Stackebrandt, E., Witt, D., Kemmerling, C., Kroppenstedt, R., Liesack, W., 1991. Designation of Streptomycete 16s and 23s Ribosomal-Rna-Based Target Regions for Oligonucleotide Probes. Appl Environ Microb 57, 1468-1477.

Stones, D.H., Krachler, A.M., 2015. Fatal Attraction: How Bacterial Adhesins Affect Host Signaling and What We Can Learn from Them. Int J Mol Sci 16, 2626-2640.

Suarez-Estrella, F., Arcos-Nievas, M.A., Lopez, M.J., Vargas-Garcia, M.C., Moreno, J., 2013. Biological control of plant pathogens by microorganisms isolated from agro-industrial composts. Biol Control 67, 509-515.

Subbarao, K.V., 1998. Progress toward integrated management of lettuce drop. Plant Dis 82, 1068-1078.

Suleman, P., Al-Musallam, A., Menezes, C.A., 2002. The effect of biofungicide Mycostop on Ceratocystis radicicola, the causal agent of black scorch on date palm. Biocontrol 47, 207-216.

Sun, J.H., Kelemen, G.H., Fernandez-Abalos, J.M., Bibb, M.J., 1999. Green fluorescent protein as a reporter for spatial and temporal gene expression in Streptomyces coelicolor A3(2). Microbiol-Uk 145, 2221-2227.

Sun, K., Liu, J., Gao, Y.Z., Jin, L., Gu, Y.J., Wang, W.Q., 2014. Isolation, plant colonization potential, and phenanthrene degradation performance of the endophytic bacterium Pseudomonas sp Ph6-gfp. Sci Rep-Uk 4.

Taechowisan, T., Peberdy, J.F., Lumyong, S., 2003. Isolation of endophytic actinomycetes from selected plants and their antifungal activity. World J Microb Biot 19, 381-385.

Takahashi, Y., Matsumoto, A., Seino, A., Ueno, J., Iwai, Y., Omura, S., 2002. Streptomyces avermectinius sp nov., an avermectin-producing strain. Int J Syst Evol Micr 52, 2163-2168.

Tamietti, G., Valentino, D., 2001. SOIL SOLARIZATION: A USEFUL TOOL FOR CONTROL OF VERTICILLIUM WILT AND WEEDS IN EGGPLANT CROPS UNDER PLASTIC IN THE PO VALLEY. J Plant Pathol 83, 173-180.

Tjamos, E.C., Tjamos, S.E., Antoniou, P.P., 2010. Biological Management of Plant Diseases: Highlights on Research and Application. J Plant Pathol 92, S17-S21.

Van Beneden, S., Pannecoucque, J., Debode, J., De Backer, G., Hofte, M., 2009. Characterisation of fungal pathogens causing basal rot of lettuce in Belgian greenhouses. Eur J Plant Pathol 124, 9-19.

Van Den Bergh, J.C.J.M., Rietveld, P., 2004. Reconsidering the limits to world population: Meta-analysis and meta-prediction. Bioscience 54, 195-204.

van Lenteren, J.C., 2005. Early entomology and the discovery of insect parasitoids. Biol Control 32, 2-7.

Van Loon, L.C., 1997. Induced resistance in plants and the role of pathogenesis-related proteins. Eur J Plant Pathol 103, 753-765.

VanOverbeek, L.S., VanVeen, J.A., VanElsas, J.D., 1997. Induced reporter gene activity, enhanced stress resistance, and competitive ability of a genetically modified Pseudomonas fluorescens strain released into a field plot planted with wheat. Appl Environ Microb 63, 1965-1973.

Velivelli, S.L., De Vos, P., Kromann, P., Declerck, S., Prestwich, B.D., 2014. Biological control agents: from field to market, problems, and challenges. Trends in biotechnology 32, 493-496.

Vessey, J.K., 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255, 571-586.

Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Marra, R., Woo, S.L., Lorito, M., 2008. Trichoderma-plant-pathogen interactions. Soil Biol Biochem 40, 1-10.

Visca, P., Colotti, G., Serino, L., Verzili, D., Orsi, N., Chiancone, E., 1992. Metal Regulation of Siderophore Synthesis in Pseudomonas-Aeruginosa and Functional-Effects of Siderophore-Metal Complexes. Appl Environ Microb 58, 2886-2893.

Waksman, S.A., Henrici, A.T., 1943. The Nomenclature and Classification of the Actinomycetes. J Bacteriol 46, 337-341.

Walsh, U.F., Morrissey, J.P., O'Gara, F., 2001. Pseudomonas for biocontrol of phytopathogens: from functional genomics to commercial exploitation. Curr Opin Biotech 12, 289-295.

Wang, J., Soisson, S.M., Young, K., Shoop, W., Kodali, S., Galgoci, A., Painter, R., Parthasarathy, G., Tang, Y.S., Cummings, R., Ha, S., Dorso, K., Motyl, M., Jayasuriya, H., Ondeyka, J., Herath, K., Zhang, C.W., Hernandez, L., Allocco, J., Basilio, A., Tormo, J.R., Genilloud, O., Vicente, F., Pelaez, F., Colwell, L., Lee, S.H., Michael, B., Felcetto, T., Gill, C., Silver, L.L., Hermes, J.D., Bartizal, K., Barrett, J., Schmatz, D., Becker, J.W., Cully, D., Singh, S.B., 2006a. Platensimycin is a selective FabF inhibitor with potent antibiotic properties. Nature 441, 358-361.

Wang, K.H., McSorley, R., Kokalis-Burelle, N., 2006b. Effects of cover cropping, solarization, and soil fumigation on nematode communities. Plant Soil 286, 229-243.
Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16s Ribosomal DNA Amplification for Phylogenetic Study. J Bacteriol 173, 697-703.

Weyens, N., Boulet, J., Adriaensen, D., Timmermans, J.P., Prinsen, E., Van Oevelen, S., D'Haen, J., Smeets, K., van der Lelie, D., Taghavi, S., Vangronsveld, J., 2012. Contrasting colonization and plant growth promoting capacity between wild type and a gfp-derative of the endophyte Pseudomonas putida W619 in hybrid poplar. Plant Soil 356, 217-230.

Wilson, K.J., 1995. Molecular Techniques for the Study of Rhizobial Ecology in the Field. Soil Biol Biochem 27, 501-514.

Xiao, K., Kinkel, L.L., Samac, D.A., 2002. Biological control of Phytophthora root rots on alfalfa and soybean with Streptomyces. Biol Control 23, 285-295.

Yandigeri, M.S., Meena, K.K., Singh, D., Malviya, N., Singh, D.P., Solanki, M.K., Yadav, A.K., Arora, D.K., 2012. Drought-tolerant endophytic actinobacteria promote growth of wheat (Triticum aestivum) under water stress conditions. Plant Growth Regul 68, 411-420.

Yedidia, I., Shoresh, M., Kerem, Z., Benhamou, N., Kapulnik, Y., Chet, I., 2003. Concomitant induction of systemic resistance to Pseudomonas spingae pv. lachrymans in cucumber by Trichoderma asperellum (T-203) and accumulation of phytoalexins. Appl Environ Microb 69, 7343-7353.

Yuan, W.M., Crawford, D.L., 1995. Characterization of Streptomyces-Lydicus Wyec108 as a Potential Biocontrol Agent against Fungal Root and Seed Rots. Appl Environ Microb 61, 3119-3128.

Appendix

Publication No.1:

Bonaldi M, Chen X*, Kunova A, Pizzatti C, Saracchi M, Cortesi P. 2015. Colonization of lettuce rhizosphere and roots by tagged *Streptomyces*. *Frontiers in Microbiology*. 6:25. *Co-first author

Publication No.2:

Chen X, Bonaldi M, Kunova A, Pizzatti C, Saracchi M, Sardi P, Cortesi P. 2015. Labeling promising biological control streptomycetes with EGFP. *IOBC-WPRS Bulletin*. In press

Publication No.3:

Bonaldi M, Chen X*, Erlacher A, Kunova A, Pizzatti C, Saracchi M, Berg, G, Cortesi P. 2015. Manuscript. *Co-first author

Publication No.4:

Bonaldi M, Saracchi M, Kunova A, Chen X, Pizzatti C, Cortesi P. Selection of *Streptomyces* against soil borne pathogens by a standardized dual culture assay and evaluation of their effects on plant growth. 2015. Submitted

Award No.1:

Poster Award of the XIII Conference of the International Organization of Biological Control on Plant Disease

Award No.2:

Winner of the "GIOVANI IN FORMAZIONE (Young Researcher)" Grant of XXI Meeting of Italian Phytopathological Society

frontiers in MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 06 February 2015
doi: 10.3389/fmicb.2015.00025

Colonization of lettuce rhizosphere and roots by tagged *Streptomyces*

Maria Bonaldi†, Xiaoyulong Chen†, Andrea Kunova†, Cristina Pizzatti†, Marco Saracchi and Paolo Cortesi*

Department of Food, Environmental and Nutritional Sciences, University of Milan, Milan, Italy

Edited by:
Aurelio Ciancio, Consiglio Nazionale delle Ricerche – Istituto per la Protezione Sostenibile delle Piante, Italy

Reviewed by:
Elena Prats, Spanish National Research Council, Spain
Stephane Compant, Austrian Institute of Technology GmbH, Austria

***Correspondence:**
Paolo Cortesi, Department of Food, Environmental and Nutritional Sciences, University of Milan, Via Giovanni Celoria, 2 20133 Milano, Italy
e-mail: paolo.cortesi@unimi.it

†These authors have contributed equally to this work.

Beneficial microorganisms are increasingly used in agriculture, but their efficacy often fails due to limited knowledge of their interactions with plants and other microorganisms present in rhizosphere. We studied spatio-temporal colonization dynamics of lettuce roots and rhizosphere by genetically modified *Streptomyces* spp. Five *Streptomyces* strains, strongly inhibiting *in vitro* the major soil-borne pathogen of horticultural crops, *Sclerotinia sclerotiorum*, were transformed with pIJ8641 plasmid harboring an enhanced green fluorescent protein marker and resistance to apramycin. The fitness of transformants was compared to the wild-type strains and all of them grew and sporulated at similar rates and retained the production of enzymes and selected secondary metabolites as well as *in vitro* inhibition of *S. sclerotiorum*. The tagged ZEA17I strain was selected to study the dynamics of lettuce roots and rhizosphere colonization in non-sterile growth substrate. The transformed strain was able to colonize soil, developing roots, and rhizosphere. When the strain was inoculated directly on the growth substrate, significantly more t-ZEA17I was re-isolated both from the rhizosphere and the roots when compared to the amount obtained after seed coating. The re-isolation from the rhizosphere and the inner tissues of surface-sterilized lettuce roots demonstrated that t-ZEA17I is both rhizospheric and endophytic.

Keywords: biocontrol, *Lactuca sativa*, *Sclerotinia sclerotiorum*, streptomycetes, rhizosphere competence

INTRODUCTION

Roots anchor plants in soil, provide uptake of water and nutrients, and mediate numerous interactions with soil organisms. The interface between roots and soil – where most of these interactions take place – is called rhizosphere. This narrow and specific zone is distinct from bulk soil in terms of nutrient availability, pH and presence of a wide variety of microorganisms and invertebrates attracted and influenced by root exudates and rhizodeposits (Hinsinger et al., 2009; Compant et al., 2010; Philippot et al., 2013). Many microbes present in rhizosphere have neutral effect on plants, while others positively or negatively affect host development and health via complex interactions, which we are only beginning to understand (Raaijmakers et al., 2009; Compant et al., 2010; Glick, 2012). Some microorganisms are deleterious as they compete with plants for nutrients or cause disease (soil borne plant pathogens), while others support their hosts by mobilizing nutrients, stimulating growth, and increasing yield or reducing biotic and abiotic stresses, such as mycorrhizal fungi and plant growth promoting bacteria (PGPB; Compant et al., 2010; Aeron et al., 2011; Smith and Smith, 2011).

Plant growth promoting bacteria are gaining more and more attention in modern agriculture, where sustainable and environmentally friendly strategies of crop cultivation increasingly rely on their use as biofertilizers, phytostimulants, or biopesticides. They employ several mechanisms to improve the plant growth, such as synthesis of phytohormones, nitrogen fixation and increasing availability of nutrients by production of siderophores and solubilization of phosphates (Lugtenberg et al., 2002; Compant et al., 2010). Furthermore, special attention is dedicated to biological control agents (BCAs), a group of microorganisms producing a wide variety of biologically active molecules potentially able to inhibit plant pathogens. Antagonism is one of the most common modes of action; here the BCA inhibits or kills pathogens via production of diffusible or volatile antimicrobial compounds and cell wall degrading enzymes. Antagonism is widespread in *Bacillus*, *Pseudomonas*, and *Streptomyces* spp., from which a wide range of biologically active secondary metabolites were isolated (Raaijmakers et al., 2002; Compant et al., 2005).

Despite the optimal performance at laboratory-scale screening tests, PGPB often fail to demonstrate their potential or show inconsistent results in greenhouse and field trials. This variable performance may have different causes, such as reduced or delayed expression of bioactive molecules in the presence of competing microorganisms or lower rhizosphere competence, i.e., poor colonization of root tissues and rhizosphere of the host plant (Lugtenberg et al., 2001; Compant et al., 2010; Ghirardi et al., 2012). To overcome these obstacles, it is essential to understand how PGPB interact with the host plant and with other microorganisms present in soil. Several studies have demonstrated better plant protection when bioactive *Pseudomonas* spp. strains with improved rhizosphere-competence were used (Ghirardi et al., 2012). In recent years, several characters essential for rhizosphere colonization were identified in *Pseudomonas*

spp. (Latour et al., 2003; Lugtenberg and Kamilova, 2009), however, similar studies are missing for other beneficial genera of bacteria.

One of such genera is *Streptomyces*, filamentous Gram-positive bacteria commonly inhabiting soil and rhizosphere and renowned for the production of a variety of bioactive secondary metabolites (Loria et al., 2006; Hopwood, 2007). They have been largely exploited in pharmaceutical industry since 1940s (Watve et al., 2001; Lucas et al., 2013), whereas only a few have been developed as commercial products for plant application in agriculture (Yuan and Crawford, 1995; Minuto et al., 2006; Berg, 2009). Streptomycetes have been long considered simply as free-living soil inhabitants, but recently the importance of their complex interactions with plants and other organisms is being uncovered (Seipke et al., 2012). Some of them, such as *S. scabies* or *S. turgidiscabies,* are plant pathogens with broad host range, causing important economic losses especially on tap root and tuber crops, such as potatoes, sweet potatoes, carrots, or beet (Loria et al., 2006; Seipke et al., 2012). On the contrary, many others establish beneficial relationships with host plants as endophytes (Sardi et al., 1992; Coombs and Franco, 2003; Cao et al., 2004). Auxin production was described for endophytic and free living *Streptomyces* in rhizosphere (Coombs et al., 2004; Khamna et al., 2009), while *S. lydicus* augmented the nodulation by *Rhizobium* species in pea plants, increasing iron and molybdenum assimilation as well as root growth (Tokala et al., 2002; Seipke et al., 2012).

Several markers have been developed and adopted to study localization and quantification of PGPB in the rhizosphere; among these, antibiotic resistance has been widely used (Prosser, 1994; Gamalero et al., 2003). Because many of soil microorganisms produce a variety of different antibiotics, it is necessary to determine the specificity of the antibiotic marker selected for the identification of PGPB before its use. Currently, fluorescent markers are gaining increasing popularity for colonization studies (Lu et al., 2004; Cao et al., 2011; Krzyzanowska et al., 2012). Various derivatives of green fluorescent protein (GFP) have been engineered to increase the fluorescence and to overcome the variable expression of the original marker in different species (Errampalli et al., 1999; Gamalero et al., 2003). Enhanced GFP (EGFP) contains numerous silent nucleotide changes in comparison to GFP to maximize its expression in mammalian cells (Haas et al., 1996), and was adopted for use in *Streptomyces* spp., which have a similar codon usage (Sun et al., 1999).

Green fluorescent protein has been utilized to study PGPB colonization of roots and rhizosphere in sterile conditions (Coombs and Franco, 2003; Weyens et al., 2012). These studies provide a basic understanding of the interactions between PGPB and the host plant, but they do not consider the complex interactions *in vivo*. In non-sterile conditions with high microbial diversity, PGPB have to compete with other microorganisms present in the rhizosphere, and in some cases the competition reduced the colonization ability of PGPB (Cao et al., 2011; Hohmann et al., 2012; Weyens et al., 2012). Moreover, the activity and the fitness of the transformed strain need to be controlled following the transformation, as it has been observed that the presence of the transgene may interfere with the biological activity of the studied organism (Nigro et al., 1999; Lübeck et al., 2002; Weyens et al., 2012).

The objective of this work was to get insight into the localization and colonization of a genetically modified *Streptomyces* strain, selected as potential BCA, in lettuce roots and rhizosphere. First, we compared the fitness of the transformed and the corresponding wild-type strains, then we studied the colonization dynamics of the most promising transformed strain in rhizosphere and roots of lettuce plants in non-sterile growth substrate. Finally, we compared the effect of two inoculation methods on the ability of the *Streptomyces* strain to differentially colonize rhizosphere and roots.

MATERIALS AND METHODS

TRANSFORMATION OF *Streptomyces* spp.

Five *Streptomyces* strains, potential BCAs against *Sclerotinia sclerotiorum*, were used in this study: CX14W, CX16W, FT05W, SW06W, and ZEA17I. They were maintained at the Plant Pathology Laboratory, Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, and selected previously from a wide collection of actinomycetes isolated from roots of different plants (Sardi et al., 1992; Petrolini et al., 1996; Bonaldi et al., 2011, 2014). *Escherichia coli* strain ET12567 (harboring the helper plasmid pUZ8002), was provided by prof. Flavia Marinelli, University of Insubria, Italy, and was used as donor strain for conjugation. Plasmid pIJ8641, obtained from prof. Mervyn Bibb, John Innes Centre, UK, was maintained in *E. coli* strain DH5α. It carries the EGFP gene under the constitutive *ermE* promoter, an apramycin resistance marker [*aac(3)IV*], an *oriT*/RK2 region, and a lambda phage chromosomal integration sequence (IntC31; Sun et al., 1999). The strain *S. coelicolor* A3(2) was obtained from F. Marinelli, and used as a reference strain to evaluate transformation efficiency.

Plasmid pIJ8641 was transformed into the donor strain *E. coli* ET12567 (pUZ8002) by rubidium chloride method (Hanahan, 1983) and conjugated into recipient *Streptomyces* strains as previously described (Kieser et al., 2000). Prior to conjugation, the concentration of the *E. coli* donor strain ET12567 containing plasmid pIJ8641 was adjusted to 1×10^7 CFU/mL. The ex-conjugants were selected on the basis of apramycin resistance. The conjugation efficiency was calculated as number of ex-conjugant colonies per number of recipient spores.

Genomic DNA of wild-type and transformed (t-) *Streptomyces* strains was extracted by the CTAB method (Kieser et al., 2000). The amplification of 16S rDNA fragment (expected size 1500 bp) was used to evaluate the quality of DNA in all samples, using PCR primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and fD2 (5′-ACGGCTACCTTGTTACGACTT-3′; Weisburg et al., 1991), and the following thermal cycling conditions: initial denaturation at 94°C for 1 min, 30 cycles of denaturation at 92°C for 45 s, annealing at 56°C for 30 s and extension at 72°C for 2 min, a final extension at 72°C for 5 min. PCR primers rEGFP-N (5′-CTGGTCGAGCTGGACGGCGACG-3′) and rEGFP-C (5′-CACGAACTCCAGCAGGACCA TG-3′) were designed to amplify the EGFP gene fragment (expected fragment size 700 bp), using the following thermal cycling conditions: initial denaturation at 94°C for 1 min, 30 cycles of denaturation at 92°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 2 min, a final

extension at 72°C for 2 min. The DNA amplification was carried out using PCR thermal cycler (BioRad, USA), performed in a total volume of 25 μL containing 30–50 ng DNA, 0.25 μM each primer, 1 U/μL Go *Taq* DNA polymerase (Promega, USA), 5 μL of 5x Go Taq buffer (Promega, USA), and 0.2 mM of each dNTP. The PCR products were visualized under Gel Doc transilluminator (BioRad, USA) following electrophoresis in 1%(w/v) agarose gel.

For microscopic observations, the transformed *Streptomyces* strains were inoculated on Czapek yeast extract agar (CZY; 35 g/L Czapek-Dox Broth Difco, 2 g/L Yeast Extract Difco, 15 g/L agar). A microscopic cover slide was partially inserted in the medium at the edge of the inoculated strain under the 45° angle to allow the strain to grow on the cover slide. The plates were incubated at 24°C for 5 days. Subsequently, cover slides were removed from the medium and observed by brightfield and epifluorescence microcopy using Olympus BX51 with the FITC filter set (467–498 nm excitation and 513–556 nm emission) to confirm the expression of EGFP in transformants.

INHIBITION OF *Sclerotinia sclerotiorum* GROWTH *IN VITRO*

The antagonistic activity of wild-type and transformed (t-) *Streptomyces* strains against *S. sclerotiorum* was determined by dual culture assay on CZY agar as described (Bonaldi et al., 2014). Briefly, 10 μL of *Streptomyces* spore suspension (1×10^7 CFU/mL) were inoculated on a 40 mm line two days prior to *S. sclerotiorum* inoculation. An agar-mycelium plug (5 mm diameter), obtained from the edge of an actively growing colony of *S. sclerotiorum* grown on Malt Extract Agar (MEA; 20 g/L Malt Extract, Difco, and 15 g/L agar), was placed at 25 mm distance from the growing *Streptomyces* colony and the plates were incubated for 72 h at 24°C. Plates inoculated with *S. sclerotiorum* only were used as a control. The antagonistic activity was determined by calculating the percentage of growth inhibition of *S. sclerotiorum* compared to the control. The experiment was repeated twice in three replicates.

MYCELIUM GROWTH AND SPORULATION

The mycelium growth curve of transformed and wild-type strains was determined daily as follows: 20 μL of *Streptomyces* spore suspension (1×10^7 CFU/mL) were transferred into a 50 mL tube containing 20 mL of CZY broth, and incubated at 30°C with 200 rpm constant shaking for 8 days. Each day, 2 mL of liquid culture were removed and spun at 10600 *g* for 10 min and the dry weight of the pellet was repeated twice in three replicates and expressed in g/L.

The sporulation of the strains was measured by plating 1 mL of spore suspension (1×10^7 CFU/mL) on a CZY agar plate and determining the number of spores produced after 6 days of incubation at 30°C (Grantcharova et al., 2005). Five mL of sterile water were added to the Petri plate and the surface of colonies was gently scraped to release the newly formed spores (Kieser et al., 2000). The spore suspension was filtrated through two layers of sterile gauze and the spore concentration (CFU/mL) was quantified by plating serial dilutions of the spore suspension and counting the number of colonies grown after 4 days of incubation at 30°C. The experiment was repeated twice in three replicates.

PRODUCTION OF SECONDARY METABOLITES

Siderophore production

Ten milliliter of Fe-free Czapek solution (300 g/L $NaNO_3$, 50 g/L KCl, 50 g/L $MgSO_4 \cdot 7H_2O$) were mixed with 15 g/L agar, 30 g/L sucrose, 1 g/L K_2HPO_4, and 5 g/L yeast extract to prepare the Fe-free Czapek agar medium. Ten microliter of *Streptomyces* spore suspension (1×10^7 CFU/mL) were inoculated in the center of a Fe-free Czapek agar plate and incubated at 30°C for two weeks. Subsequently, the *Streptomyces* colony was overlaid by 15 mL of the Chrome azurol S (CAS) agar (Schwyn and Neilands, 1987; Perez-Miranda et al., 2007). The siderophore production was determined as color change in the overlay medium (from blue to orange) after 24 h of incubation at room temperature. The experiment was repeated twice in three replicates.

Chitinase production

The colloidal chitin and the colloidal chitin agar were prepared as described previously (Saima et al., 2013). Ten microliter of *Streptomyces* spore suspension (1×10^7 CFU/mL) were inoculated in the center of colloidal chitin agar plate (chitin as single carbon sources) as a 40 mm line and incubated at 30°C for 10 days. The production of chitinase was determined based on the presence of a clear hydrolysis zone on the agar plate below the colony. The experiment was repeated twice in three replicates.

Phosphate solubilization

The phosphate solubilization activity of the *Streptomyces* strains was assessed using a plate assay with National Botanical Research Institute's Phosphate (NBRIP) medium (Nautiyal, 1999), in which $Ca_3(PO_4)_2$ is the only phosphate source. Ten microliter of *Streptomyces* spore suspension (1×10^7 CFU/mL) were inoculated in the center of a NBRIP-medium Petri plate and incubated at 30°C for 2 weeks. The phosphate solubilization was determined based on the presence of a clear hydrolysis zone on the agar plate below the colony. The test was repeated twice in three replicates.

Indole-3-acetic acid (IAA) synthesis

The IAA production was determined as described previously (Bric et al., 1991; Bano and Musarrat, 2003). In brief, 10 μL of *Streptomyces* spore suspension (1×10^7 CFU/ml) were incubated with constant shaking at 5 *g* in 5 mL CZY broth added with 500 μg/mL tryptophan (Sigma, USA) in the dark at 30°C for 10 days. Two mL of the liquid culture were centrifuged for 10 min at 18000 *g*. One mL of the supernatant was mixed with 50 μL 10 mM orthophosphoric acid and 2 mL of Salkowski reagent (1 mL of 0.5M $FeCl_3$ in 50 mL of 35% $HClO_4$). The tubes were incubated at room temperature for 30 min. The development of pink color indicated the IAA production, which was quantified by spectrophotometer at 530 nm. The experiment was repeated twice in three replicates.

SOIL, ROOT, AND RHIZOSPHERE COLONIZATION BY t-ZEA17I

The transformed ZEA17I strain (t-ZEA17I) was grown on CZY medium containing 50 mg/L of apramycin at 24°C for 10 days. Spores were collected in 10% sterile glycerol and filtered through two layers of gauze. The concentration was determined and the spore suspension was stored at −20°C.

Bulk soil colonization

Prior to colonization studies, the presence of naturally occurring apramycin-resistant streptomycetes in non-sterilized Irish and Baltic peat-based growth substrate (Vigorplant, Italy) was assessed. A portion of the substrate was resuspended in sterile water and plated on water agar medium (WA) containing 15 g/L agar, 25 mg/L nalidixic adic, 50 mg/L apramycin, 50 mg/L nystatin, and 50 mg/L cycloheximide. Plates were incubated for 7 days at 24°C and the presence of apramycin-resistant streptomycete colonies was visually checked.

The growth substrate was placed in a polystyrene seed tray (48 cm^3/cell) and watered with tap water. In every cell, t-ZEA17I was uniformly distributed on the top of the substrate adding 1 mL spore suspension (1×10^7 CFU/mL). The growth substrate was incubated in a growth chamber (24°C, 55% relative humidity and 15 h photoperiod) and watered every 2–3 days with tap water. t-ZEA17I was re-isolated 4 h (day 0), 10, 20, and 30 days after inoculation (dai) in six replicates. The entire amount of growth substrate in the cell was collected and weighed. The substrate was mixed to homogenize the inoculum and divided in two identical parts. One part was incubated in the oven at 50°C and the dry weight was determined. The other part was stirred in sterilized water (1:10 substrate fresh w/v) for one hour and serial dilutions were plated on WA. Plates were incubated for 7 days at 24°C and streptomycete colonies were counted. The t-ZEA17I concentration was expressed as CFU/g of growth substrate dry weight.

Plant inoculation

Ice queen lettuce seedlings (*Lactuca sativa* var. *capitata*, Iceberg group, Semeurop, Italy) were grown in polystyrene seed trays, as described previously. Seeds were surface sterilized in 0.7% sodium hypochlorite for 5 min and rinsed three times in sterile water. Two methods were used to inoculate the t-ZEA17I strain. In the growth substrate inoculation method, 1 mL spore suspension (1×10^7 CFU/mL) was uniformly distributed in every cell on the top of the growth substrate. In the seed coating method, 50 seeds were soaked in 1 mL of t-ZEA17I spore suspension (1×10^7 CFU/mL) and left to dry under the laminar flow hood. One seed for each cell of the tray was sown and the seedlings were incubated and watered as described previously.

To determine the inoculum load t-ZEA17I was re-isolated from seeds and growth substrate after inoculum application. In case of the growth substrate inoculation method, the t-ZEA17I strain was re-isolated four hours after soil inoculation as described above for bulk soil, and its amount was expresses as CFU/g of growth substrate dry weight. For the seed coating method, 10 randomly collected seeds were incubated for 30 min in 1 mL of sterile 0.9% NaCl and serial dilutions were plated on WA medium in six replicates. Following incubation at 24°C for 7 days the t-ZEA17I colonies were counted and the amount was expressed first as CFU/seed and then recalculated as CFU/g of growth substrate dry weight.

t-ZEA17I re-isolation from rhizosphere and root tissues

The t-ZEA17I strain was re-isolated 10, 20, and 30 days after sowing from rhizosphere and root tissues of six lettuce seedlings, equal to number of replicates. Seedlings with root system were carefully taken off the cell and the bulk soil was removed by gently shaking the plants (Bulgarelli et al., 2012).

For the rhizosphere analysis, each seedling was cut at base and the roots were vortexed two times for 15 s in 1–3 mL (volume varying according to period of sampling) of sterilized 0.9% NaCl and 0.02% Silwet L-77 (washing solution). The roots were removed and the suspension was filtered through a 300 μm nylon mesh to obtain the rhizosphere soil and its dry weight was determined. The suspension was centrifuged at 10600 *g* for 10 min and the pellet was resuspended in 0.5–1.5 mL of washing solution and plated in serial dilutions on WA medium. The plates were incubated at 24°C for 7 days. The t-ZEA17I colonies were counted and the concentration was expressed as CFU/g of rhizosphere dry weight.

For inner root tissues analysis, the roots were surface sterilized with propylene oxide for one hour (Sardi et al., 1992). Then, they were washed in washing solution and 1/10 of the total volume was plated on WA medium to verify the absence of contaminants. Subsequently the roots were finely homogenized in 1–3 mL washing solution, let to macerate for one hour and the suspension was plated in serial dilutions. The t-ZEA17I concentration was determined as described before and expressed as CFU/g of roots dry weight.

STATISTICAL ANALYSES

All analyses were done using R software, version R3.0.2. (R Core Team, 2013). The statistical differences between data of transformed and wild-type strains in *S. sclerotiorum* growth inhibition, sporulation, and IAA production were compared by a Student's *t*-test ($P = 0.05$). The percent data were arcsine root-squared transformed. The soil, root, and rhizosphere colonization data were submitted to ANOVA, followed by a Tukey *post hoc* test for multiple comparison ($P = 0.05$), using the TukeyC package (Faria et al., 2013).

RESULTS

TRANSFORMATION OF *Streptomyces* spp. WITH PLASMID pIJ8641

All six strains, including *S. coelicolor* A3(2) were transformed with the pIJ8641 plasmid harboring the EGFP gene under a constitutive promoter and apramycin resistance. Conjugation efficiency varied among strains: four strains, CX14W, SW06W, CX16W, and FT05W showed conjugation efficiency similar to *S. coelicolor* A3(2), while one strain, ZEA17I conjugated with lower efficiency (**Table 1**). The EGFP gene was detected in transformed strains (data not shown), and its expression was confirmed by fluorescence microscopy observing a strong green fluorescence in all transformants following exposition to fluorescent light (**Figure 1**), while the corresponding wild-type strains did not fluoresce.

EFFECT OF THE TRANSFORMATION ON STRAIN FITNESS

Following the transformation, the fitness of transformants was evaluated in terms of mycelium growth and sporulation, inhibition of *S. sclerotiorum* mycelium growth, and production of selected secondary metabolites. All the transformed strains showed similar mycelium growth and sporulation to their corresponding wild-type strains (**Figure 2** – for simplicity, the growth

Table 1 | Conjugation efficiencies of five *Streptomyces* spp. strains and *S. coelicolor* A3(2) with the pIJ8641 plasmid.

Strain	Recipient strain (CFU/mL)	Conjugation efficiency (CFU/recipient strain)
S. coelicolor A3(2)	1×10^{8}	9.10×10^{-6}
CX14W	1×10^{8}	4.64×10^{-5}
CX16W	1×10^{10}	6.88×10^{-6}
FT05W	1×10^{9}	1.60×10^{-6}
SW06W	1×10^{8}	3.13×10^{-5}
ZEA17I	1×10^{9}	5.81×10^{-8}

curves for only two strains were reported, **Table 2**). The transformants inhibited *S. sclerotiorum* radial growth from 66 to 81%, and no significant differences were observed between wild-type and transformed strains. Finally, no significant differences were detected in siderophore, auxin, chitinase production, and phosphate solubilization (**Tables 2** and **3**).

COLONIZATION DYNAMICS OF THE TRANSFORMED ZEA17I IN BULK SOIL

In this study, apramycin was used as a marker to identify the transformed t-ZEA17I strain. However, to be able to use it in greenhouse experiments, we first checked for the presence of naturally occurring apramycin-resistant *Streptomyces* spp. in the growth substrate, but none were detected. Therefore non-sterilized growth substrate was used in following experiments.

The colonization dynamics of t-ZEA17I in bulk soil showed that the initial inoculum, 1.16×10^{8} CFU/g dry weight added to the soil, was recovered at 3.85×10^{7} CFU/g dry weight four hours after inoculation (0 dai). The t-ZEA17I amount decreased significantly within the first 10 days and thereafter it remained stable up to 30 days at 2.18×10^{4} CFU/g dry weight (**Table 4**).

COLONIZATION OF LETTUCE RHIZOSPHERE AND INNER ROOT TISSUES BY THE TRANSFORMED ZEA17I

The *Streptomyces* strain t-ZEA17I was inoculated with two different methods: as a spore suspension distributed on soil surface and as seed coating. The colonization dynamics of rhizosphere and

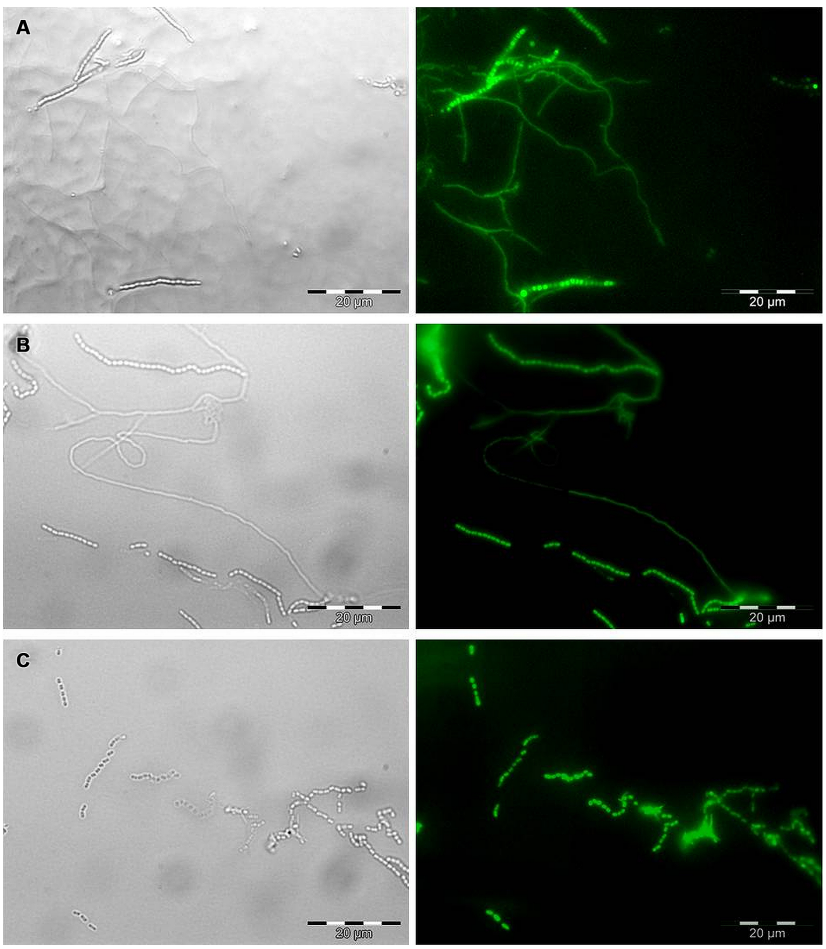


FIGURE 1 | Microscopic observation of EGFP expression in transformed *Streptomyces* strains by bright field (left) and epifluorescence microscopy (right). Mycelium and spore chains of **(A)** the strain t-ZEA17I, **(B)** the strain t-FT05W, and **(C)** spore chains of the strain t-CX16W. Scale bar, 20 µm.

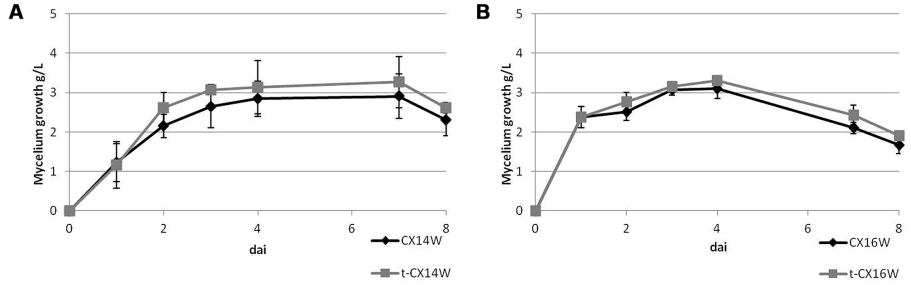


FIGURE 2 | Mycelium growth of the wild-type and transformed *Streptomyces* spp. strains, (A) CX14W and (B) CX16W. Vertical bars represent SE ($N = 3$).

Table 2 | Sporulation, auxin production, and inhibition of *Sclerotinia sclerotiorum* mycelium growth of the wild-type and transformed (t-) *Streptomyces* spp.

Strain	Sporulation (10^9 CFU/mL)		Auxin production (μg/mL)		*S. sclerotiorum* inhibition (%)	
CX14W	7.47 ± 3.22[1]	$P^2 = 0.229$	5.82 ± 0.44	$P = 0.278$	78.80 ± 1.70	$P = 0.072$
t-CX14W	6.80 ± 4.91		5.11 ± 0.35		73.00 ± 2.28	
CX16W	4.23 ± 6.58	$P = 0.178$	5.87 ± 0.86	$P = 0.872$	68.99 ± 0.98	$P = 0.091$
t-CX16W	5.55 ± 1.61		6.03 ± 0.24		65.87 ± 1.33	
FT05W	1.45 ± 3.69	$P = 0.242$	6.04 ± 0.60	$P = 0.207$	67.59 ± 0.68	$P = 0.684$
t-FT05W	1.37 ± 4.56		7.11 ± 0.21		67.13 ± 0.85	
SW06W	2.27 ± 1.15	$P = 0.205$	7.32 ± 0.66	$P = 0.588$	80.21 ± 1.34	$P = 1.000$
t-SW06W	0.78 ± 1.67		6.88 ± 0.28		80.21 ± 1.34	
ZEA17I	0.47 ± 0.23	$P = 0.417$	7.56 ± 0.59	$P = 0.690$	74.80 ± 1.76	$P = 0.069$
t-ZEA17I	1.63 ± 1.15		6.84 ± 0.38		80.75 ± 1.51	

[1] *Mean value followed by SE.* [2] *P-values of the Student's t-test pairwise comparisons.*

Table 3 | Phosphate solubilization, chitinase, and siderophore production of the wild-type and transformed (t-) *Streptomyces* spp.

Strain	Phosphate solubilization	Chitinase production	Siderophore production
CX14W	+[1]	+	+
t-CX14W	+	+	+
CX16W	+	+	+
t-CX16W	+	+	+
FT05W	+	+	−
t-FT05W	+	+	−
SW06W	+	+	−
t-SW06W	+	+	−
ZEA17I	+	+	−
t-ZEA17I	+	+	−

[1] + *indicates activity.* − *indicates no activity.*

inner root tissues of lettuce seedlings differed between the two methods.

In the rhizosphere, the concentration of the t-ZEA17I strain remained similar to the inoculated amount during the first 20 dai with either method. When t-ZEA17I was distributed on top of the growth substrate, a significant increase in concentration 30 dai was observed. In the case of the seed coating, after a slight increase within the first 10 days, the final amount was not significantly different from the initial inoculum (**Table 5**).

Similarly, we studied the dynamics of t-ZEA17I colonization in the inner tissues of lettuce roots. First, we ruled out the possible external root contamination due to ineffective sterilization, and no *Streptomyces* colonies were detected. The t-ZEA17I strain was re-isolated from inner root tissues of surface-sterilized roots independently of the inoculation method, confirming its ability to endophytically colonize lettuce roots. The concentration of t-ZEA17I declined steadily through time, however, this reduction was not significant with either inoculation method (**Table 6**).

Finally, we compared the two inoculation methods to get a further insight into whether one of them could improve the survival and colonization rates of t-ZEA17I in lettuce rhizosphere and roots. In the rhizosphere, significantly more t-ZEA17I was re-isolated at all sampling times using the growth substrate inoculation rather than seed coating ($P = 0.0037$, 0.0389, and 0.0005, for sampling time 10, 20, and 30 dai, respectively). Similarly, in roots, significantly higher concentration of t-ZEA17I was re-isolated using the growth substrate inoculation at 20 and 30 dai

Table 4 | Colonization dynamics in bulk soil by transformed *Streptomyces* ZEA17I.

Inoculation method	Bulk soil (CFU/g dry weight)			
	0 dai[1]	10 dai	20 dai	30 dai
Growth substrate	3.85×10^7a[2]	1.25×10^4b	1.54×10^4b	2.18×10^4b

[1] *dai = days after inoculation.* [2] *Tukey post hoc test; means in a row with the same letters are not significantly different (P = 0.05).*

Table 5 | Colonization dynamics of *Lactuca sativa* var. *capitata* rhizosphere by transformed *Streptomyces* ZEA17I strain according to two inoculation methods.

Inoculation method	Rhizosphere (CFU/g dry weight)			
	0 dai[1]	10 dai	20 dai	30 dai
Growth substrate	2.51×10^6 b[2]	2.72×10^7 ab	3.07×10^7 a	3.80×10^7 a
Seed coating	1.28×10^6 ab	2.01×10^6 a	9.85×10^5 ab	1.19×10^5 b

[1] *dai = days after inoculation.* [2] *Tukey post hoc test; means in a row with the same letters are not significantly different (P = 0.05).*

Table 6 | Colonization dynamics of *L. sativa* var. *capitata* inner root tissues by transformed *Streptomyces* ZEA17I strain according to two inoculation methods.

Inoculation method	Roots (CFU/g dry weight)		
	10 dai[1]	20 dai	30 dai
Growth substrate	1.94×10^7 ns[2]	1.45×10^6 ns	2.36×10^5 ns
Seed coating	3.93×10^5 ns	2.23×10^5 ns	1.39×10^4 ns

[1] *dai = days after inoculation.* [2] *ns, ANOVA not significant (P > 0.05).*

($P = 0.0415$ and $P = 0.0604$, respectively). However, in spite of higher strain amounts present in roots using the growth substrate inoculation method, not all seedlings were colonized. Indeed, we failed to re-isolate t-ZEA17I from roots of three seedlings (one seedling at 20 dai and two seedlings at 30 dai), whereas, using the seed coating method, all roots were endophytically colonized.

DISCUSSION

Plant beneficial bacteria have a great potential in agriculture as PGPB and BCAs and reports about successful control of plant diseases are increasing. However, application of these microbial agents in field often fails to achieve the expected results, which could be due to lack of knowledge about their biology and interactions with the host plant, the pathogens, and other microorganisms in the rhizosphere. Therefore, there are increasing attempts to study these complex interactions that take place in the rhizosphere (Gamalero et al., 2003; Compant et al., 2010).

Our aim was to study spatio-temporal dynamics of colonization of lettuce roots and rhizosphere by *Streptomyces* spp. with biological control potential, to better understand if and how they inhabit the rhizosphere and colonize plant roots. We selected five *Streptomyces* strains on the basis of their strong *in vitro* antagonism against the major soil-borne pathogen of horticultural crops, *S. sclerotiorum* (Bonaldi et al., 2014), and we transformed them with the pIJ8641 plasmid harboring apramycin resistance marker and EGFP gene under a strong constitutive promoter (Sun et al., 1999). The conjugation efficiency varied, and for most strains it was comparable to the reference strain *S. coelicolor* A3(2). The pIJ8641 plasmid integrates at the chromosomal attachment site for the temperate phage φC31, which may result in disruption or alteration of fitness and biological activity of the transformed strains. Indeed, decrease or loss of biological activity was detected after GFP-transformation of various BCAs, e.g., *Pseudomonas putida*, *Metschnikowia pulcherrima,* or *Clonostachys rosea* (Nigro et al., 1999; Lübeck et al., 2002; Weyens et al., 2012). We compared several traits important for biological control and plant growth promotion of transformed and wild-type strains, before studying their interactions with the host plant. None of the transformed strains showed altered growth or sporulation, which could have conferred a disadvantage in plant root and rhizosphere colonization. All transformants retained the ability to suppress growth of *S. sclerotiorum in vitro*, therefore they will also be used for studying their interactions with the pathogen and the mechanisms of biological control *in vivo*. Moreover, we compared the expression of some of the most common traits involved in plant growth promotion and biological control (Brader et al., 2014), such as production of auxins, siderophores and lytic enzymes, and no change in performance between the wild type and the transformants was observed.

We chose the most promising transformed strain, t-ZEA17I, for pilot studies of lettuce roots and rhizosphere colonization. We intentionally used a non-sterile growth substrate to simulate competition with natural microflora and evaluate the competitiveness of the inoculated *Streptomyces* strain exploiting the apramycin resistance for its identification among soil microorganisms. In absence of the host plant, we confirmed that t-ZEA17I freely survives in soil, although we observed a sharp decrease in its density

within the first 10 days after bulk soil inoculation. Similar dynamics for introduced microbial population in non-sterile soil are already known, attributed to scarcity of available nutrients and adverse biotic and abiotic factors (van Veen et al., 1997). However, following the initial fall in population density, the t-ZEA17I population remained stable for up to 30 days, probably establishing an equilibrium with the indigenous microflora as described previously (Yuan and Crawford, 1995; Merzaeva and Shirokikh, 2006). In the presence of the lettuce plant, we did not detect in the rhizosphere the initial rapid decrease in t-ZEA17I amount that was observed in bulk soil. On the contrary, its concentration augmented when applied directly on the growth substrate. It is possible that t-ZEA17I was chemoattracted to the rhizosphere of the growing seedling, where it quickly established a stable interaction with the host plant roots. Indeed, the presence of a host plant may greatly affect the survival of PGPB, as was observed, i.e., for the sharp decline in *Azospirillum brasilense* population after removal of inoculated plants (Bashan et al., 1995).

Different strategies are being used for studying BCAs and PGPB in the rhizosphere. Their localization in roots and seeds rely on microscopic tools exploiting fluorescent markers, which give a fundamental insight into the spatial distribution of the microorganism along and inside the growing root, but do not quantify the microbial amounts and their dynamics (Coombs and Franco, 2003; Olivain et al., 2006; Compant et al., 2010). Additionally, studying the dynamics of colonization by beneficial microorganisms exploits the strain identification mostly by natural or introduced antibiotic resistance and its quantification by dilution plating (Gamalero et al., 2003, 2005). Here, we quantified the t-ZEA17I in roots and rhizosphere through the introduced antibiotic resistance for its identification, to understand if it can inhabit soil in competitive concentrations in comparison to the indigenous microflora. t-ZEA17I was detected in the inner root tissues of growing seedlings already 10 days after inoculation at high concentrations. Indeed, Coombs and Franco (2003) demonstrated that the EGFP-tagged endophytic *Streptomyces* sp. strain EN27 rapidly colonizes the wheat embryo, as it was detected in developing roots as early as 24 h after inoculation. Re-isolation of t-ZEA17I from the rhizosphere and the inner tissues of surface-sterilized roots indicates that it is both rhizospheric and endophytic, although it is not known if its localization affects its potential for biocontrol and plant growth promotion. It has been hypothesized that endophytic bacteria form more stable interactions with plants, rather than rhizospheric or epiphytic bacteria (Compant et al., 2010; Malfanova et al., 2011).

Finally, we tested how different methods of inoculation influence the t-ZEA17I colonization ability. When it was distributed directly on the growth substrate, higher concentration was detected in roots, however, we could not re-isolate the strain from all inoculated plants. On the contrary, when the seed coating method was used, less propagules were recovered but all plants were endophytically colonized. It is possible that in case of seed coating, t-ZEA17I is more closely associated with the growing seedling, which increases its probability to internally colonize root tissues. In roots, we observed progressive decline in its concentration using either inoculation method. Although the total amount of t-ZEA17I increased at different sampling times (data not shown), the increase in lettuce root biomass was probably higher than the strain growth, thus resulting in lower strain concentration per g of root. To ensure that t-ZEA17I colonizes roots in effective concentrations, and to prevent its decline to undetectable levels, studies assessing optimal amount of inoculum are needed. Moreover, it is possible that strains colonize only certain root zones (Gamalero et al., 2004, 2005). Therefore, further studies are needed to establish which zones of the plant roots are colonized by t-ZEA17I and ultimately how it interacts with the plant in presence of *S. sclerotiorum,* to evaluate its biological control activity *in vivo*.

ACKNOWLEDGMENTS

The authors thank prof. Flavia Marinelli (University of Insubria, Italy), for donor strain *E. coli* ET12567 and reference strain *S. coelicolor* A3(2) and Prof. Mervyn Bibb (John Innes Centre, UK), for kindly providing the plasmid pIJ8641. The authors also thank the reviewers for their constructive and useful comments that greatly improved the manuscript. This research was supported in part by research program "Dote ricerca applicata" funded by Lombardy Region and Sipcam Italia Spa.

REFERENCES

Aeron, A., Kumar, S., Pandey, P., and Maheshwari, D. K. (2011). "Emerging role of plant growth promoting rhizobacteria in agrobiology," in *Bacteria in Agrobiology: Crop Ecosystems*, ed. D. K. Maheshwari (Berlin: Springer), 1–36. doi: 10.1007/978-3-642-18357-7_1

Bano, N., and Musarrat, J. (2003). Characterization of a new *Pseudomonas aeruginosa* strain NJ-15 as a potential biocontrol agent. *Curr. Microbiol.* 46, 0324–0328. doi: 10.1007/s00284-002-3857-8

Bashan, Y., Puente, M. E., Rodriguezmendoza, M. N., Toledo, G., Holguin, G., Ferreracerrato, R., et al. (1995). Survival of Azospirillum brasilense in the bulk soil and rhizosphere of 23 soil types. *Appl. Environ. Microbiol.* 61, 1938–1945.

Berg, G. (2009). Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl. Microbiol. Biotechnol.* 84, 11–18. doi: 10.1007/s00253-009-2092-7

Bonaldi, M., Kunova, A., Saracchi, M., Sardi, P., and Cortesi, P. (2014). Streptomycetes as biological control agents against basal drop. *Acta Hortic.* 1044, 313–318.

Bonaldi, M., Kunova, A., Sardi, P., Cortesi, P., and Saracchi, M. (2011). Streptomycetes as possible biocontrol agents against soil-borne pathogens. *J. Plant Pathol.* 93, S4.26–S4.27.

Brader, G., Compant, S., Mitter, B., Trognitz, F., and Sessitsch, A. (2014). Metabolic potential of endophytic bacteria. *Curr. Opin. Biotechnol.* 27, 30–37. doi: 10.1016/j.copbio.2013.09.012

Bric, J. M., Bostock, R. M., and Silverstone, S. E. (1991). Rapid in situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. *Appl. Environ. Microbiol.* 57, 535–538.

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren Van Themaat, E., Ahmadinejad, N., Assenza, F., et al. (2012). Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. *Nature* 488, 91–95. doi: 10.1038/nature11336

Cao, L., Qiu, Z., You, J., Tan, H., and Zhou, S. (2004). Isolation and characterization of endophytic *Streptomyces* strains from surface-sterilized tomato (*Lycopersicon esculentum*) roots. *Lett. Appl. Microbiol.* 39, 425–430. doi: 10.1111/j.1472-765X.2004.01606.x

Cao, Y., Zhang, Z., Ling, N., Yuan, Y., Zheng, X., Shen, B., et al. (2011). Bacillus subtilis SQR 9 can control Fusarium wilt in cucumber by colonizing plant roots. *Biol. Fertility Soils* 47, 495–506. doi: 10.1007/s00374-011-05562

Compant, S., Clement, C., and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved, and prospects for utilization. *Soil. Biol. Biochem.* 42, 669–678. doi: 10.1016/j.soilbio.2009.11.024

Compant, S., Duffy, B., Nowak, J., Clement, C., and Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* 71, 4951–4959. doi: 10.1128/Aem.71.9.4951-4959.2005

Coombs, J. T., and Franco, C. M. M. (2003). Visualization of an endophytic *Streptomyces* species in wheat seed. *Appl. Environ. Microbiol.* 69, 4260–4262. doi: 10.1128/Aem.69.7.4260-4262.2003

Coombs, J. T., Michelsen, P. P., and Franco, C. M. (2004). Evaluation of endophytic actinobacteria as antagonists of *Gaeumannomyces graminis* var. tritici in wheat. *Biol. Control* 29, 359–366. doi: 10.1016/j.biocontrol.2003.08.001

Errampalli, D., Leung, K., Cassidy, M. B., Kostrzynska, M., Blears, M., Lee, H., et al. (1999). Applications of the green fluorescent protein as a molecular marker in environmental microorganisms. *J. Microbiol. Methods* 35, 187–199. doi: 10.1016/S0167-7012(99)00024-X

Faria, J. C., Jelihovschi, E. G., and Allaman, I. B. (2013). *Conventional Tukey Test.* Ilheus: Test.UESC.

Gamalero, E., Lingua, G., Berta, G., and Lemanceau, P. (2003). Methods for studying root colonization by introduced beneficial bacteria. *Agronomie* 23, 407–418. doi: 10.1051/Agro:2003014

Gamalero, E., Lingua, G., Capri, F. G., Fusconi, A., Berta, G., and Lemanceau, P. (2004). Colonization pattern of primary tomato roots by *Pseudomonas fluorescens* A6RI characterized by dilution plating, flow cytometry, fluorescence, confocal, and scanning electron microscopy. *FEMS Microbiol. Ecol.* 48, 79–87. doi: 10.1016/j.femsec.2003.12.012

Gamalero, E., Lingua, G., Tombolini, R., Avidano, L., Pivato, B., and Berta, G. (2005). Colonization of tomato root seedling by *Pseudomonas fluorescens* 92rkG5: spatio-temporal dynamics, localization, organization, viability, and culturability. *Microb. Ecol.* 50, 289–297. doi: 10.1007/s00248-004-0149-9

Ghirardi, S., Dessaint, F., Mazurier, S., Corberand, T., Raaijmakers, J. M., Meyer, J. M., et al. (2012). Identification of traits shared by rhizosphere-competent strains of fluorescent pseudomonads. *Microb. Ecol.* 64, 725–737. doi: 10.1007/s00248-012-0065-3

Glick, B. R. (2012). Plant growth-promoting bacteria: mechanisms and applications. *Scientifica* 2012, 15. doi: 10.6064/2012/963401

Grantcharova, N., Lustig, U., and Flardh, K. (2005). Dynamics of FtsZ assembly during sporulation in *Streptomyces* coelicolor A3(2). *J. Bacteriol.* 187, 3227–3237. doi: 10.1128/Jb.187.3227.3237.2005

Haas, J., Park, E.-C., and Seed, B. (1996). Codon usage limitation in the expression of HIV-1 envelope glycoprotein. *Curr. Biol.* 6, 315–324. doi: 10.1016/S0960-9822(02)00482-7

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166, 557–580. doi: 10.1016/S0022-2836(83)80284-8

Hinsinger, P., Bengough, A. G., Vetterlein, D., and Young, I. (2009). Rhizosphere: biophysics, biogeochemistry and ecological relevance. *Plant Soil* 321, 117–152. doi: 10.1007/s11104-008-9885-9889

Hohmann, P., Jones, E. E., Hill, R. A., and Stewart, A. (2012). Ecological studies of the bio-inoculant *Trichoderma* hamatum LU592 in the root system of Pinus radiata. *FEMS Microbiol. Ecol.* 80, 709–721. doi: 10.1111/j.1574-6941.2012.01340.x

Hopwood, D. A. (2007). *Streptomyces in Nature and Medicine: The Antibiotic Makers.* Oxford: Oxford University Press New York.

Khamna, S., Yokota, A., and Lumyong, S. (2009). Actinomycetes isolated from medicinal plant rhizosphere soils: diversity and screening of antifungal compounds, indole-3-acetic acid and siderophore production. *World J. Microbiol. Biotechnol.* 25, 649–655. doi: 10.1007/s11274-008-9933-x

Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000). *Practical Streptomyces Genetics.* Norwich: The John Innes Foundation.

Krzyzanowska, D., Obuchowski, M., Bikowski, M., Rychlowski, M., and Jafra, S. (2012). Colonization of potato rhizosphere by GFP-tagged *Bacillus subtilis* MB73/2, *Pseudomonas* sp. P482 and *Ochrobactrum* sp. A44 shown on large sections of roots using enrichment sample preparation and confocal laser scanning microscopy. *Sensors* 12, 17608–17619. doi: 10.3390/S121217608

Latour, X., Delorme, S., Mirleau, P., and Lemanceau, P. (2003). Identification of traits implicated in the rhizosphere competence of fluorescent pseudomonads: description of a strategy based on population and model strain studies. *Agronomie* 23, 397–405. doi: 10.1051/Agro:2003015

Loria, R., Kers, J., and Joshi, M. (2006). Evolution of plant pathogenicity in *Streptomyces. Annu. Rev. Phytopathol.* 44, 469–487. doi: 10.1146/annurev.phyto.44.032905.091147

Lu, Z. X., Tombolini, R., Woo, S., Zeilinger, S., Lorito, M., and Jansson, J. K. (2004). In vivo study of *Trichoderma*-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Appl. Environ. Microbiol.* 70, 3073–3081. doi: 10.1128/Aem.70.5.3073-3081.2004

Lübeck, M., Knudsen, I., Jensen, B., Thrane, U., Janvier, C., and Funck Jensen, D. (2002). GUS and GFP transformation of the biocontrol strain *Clonostachys rosea* IK726 and the use of these marker genes in ecological studies. *Mycol. Res.* 106, 815–826. doi: 10.1017/S095375620200607X

Lucas, X., Senger, C., Erxleben, A., Grüning, B. A., Döring, K., Mosch, J., et al. (2013). StreptomeDB: a resource for natural compounds isolated from *Streptomyces* species. *Nucleic Acids Res.* 41, D1130–D1136. doi: 10.1093/nar/gks1253

Lugtenberg, B. J., Chin-A-Woeng, T. C., and Bloemberg, G. (2002). Microbe–plant interactions: principles and mechanisms. *Antonie Van Leeuwenhoek* 81, 373–383. doi: 10.1023/a:1020596903142

Lugtenberg, B. J. J., Dekkers, L., and Bloemberg, G. V. (2001). Molecular determinants of rhizosphere colonization by *Pseudomonas. Annu. Rev. Phytopathol.* 39, 461–490. doi: 10.1146/annurev.phyto.39.1.461

Lugtenberg, B., and Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556. doi: 10.1146/annurev.micro.62.081307.162918

Malfanova, N., Kamilova, F., Validov, S., Shcherbakov, A., Chebotar, V., Tikhonovich, I., et al. (2011). Characterization of *Bacillus* subtilis HC8, a novel plant-beneficial endophytic strain from giant hogweed. *Microbial Biotechnol.* 4, 523–532. doi: 10.1111/j.1751-7915.2011.00253.x

Merzaeva, O. V., and Shirokikh, I. G. (2006). Colonization of plant rhizosphere by actinomycetes of different genera. *Microbiology* 75, 226–230. doi: 10.1134/S0026261706020184

Minuto, A., Spadaro, D., Garibaldi, A., and Gullino, M. L. (2006). Control of soilborne pathogens of tomato using a commercial formulation of *Streptomyces* griseoviridis and solarization. *Crop Prot.* 25, 468–475. doi: 10.1016/j.cropro.2005.08.001

Nautiyal, C. S. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol. Lett.* 170, 265–270. doi: 10.1111/j.1574-6968.1999.tb13383.x

Nigro, F., Finetti Sialer, M. M., and Gallitelli, D. (1999). Transformation of *Metschnikowia* pulcherrima 320, biocontrol agent of storage rot, with the green fluorescent protein gene. *J. Plant Pathol.* 205–208.

Olivain, C., Humbert, C., Nahalkova, J., Fatehi, J., L'haridon, F., and Alabouvette, C. (2006). Colonization of tomato root by pathogenic and non-pathogenic *Fusarium oxysporum* strains inoculated together and separately into the soil. *Appl. Environ. Microbiol.* 72, 1523–1531. doi: 10.1128/Aem.72.2.1523-1531.2006

Perez-Miranda, S., Cabirol, N., George-Tellez, R., Zamudio-Rivera, L. S., and Fernandez, F. J. (2007). O-CAS, a fast and universal method for siderophore detection. *J. Microbiol. Methods* 70, 127–131. doi: 10.1016/j.mimet.2007.03.023

Petrolini, B., Quaroni, S., Saracchi, M., and Sardi, P. (1996). Studies on the streptomycete population inhabiting plant roots. *Actinomycetes* 7, 66–78.

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and Van Der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789–799. doi: 10.1038/Nrmicro3109

Prosser, J. I. (1994). Molecular marker systems for detection of genetically-engineered microorganisms in the environment. *Microbiology* 140, 5–17. doi: 10.1099/13500872-140-1-5

R Core Team. (2013). *R: A Language and Environment for Statistical Computing.* (Vienna: R Foundation for Statistical Computing).

Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., Alabouvette, C., and Moenne-Loccoz, Y. (2009). The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil* 321, 341–361. doi: 10.1007/s11104-008-9568-6

Raaijmakers, J. M., Vlami, M., and De Souza, J. T. (2002). Antibiotic production by bacterial biocontrol agents. *Antonie Van Leeuwenhoek International J. Gen. Mol. Microbiol.* 81, 537–547. doi: 10.1023/A:1020501420831

Saima, M., Kuddus, M., Roohi, I., and Ahmad, Z. (2013). Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinase. *J. Genet. Eng. Biotechnol.* 11, 39–46. doi: 10.1016/j.jgeb.2013.03.001

Sardi, P., Saracchi, M., Quaroni, S., Petrolini, B., Borgonovi, G. E., and Merli, S. (1992). Isolation of endophytic *Streptomyces* strains from surface-sterilized roots. *Appl. Environ. Microbiol.* 58, 2691–2693.

Schwyn, B., and Neilands, J. B. (1987). Universal chemical-assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47–56. doi: 10.1016/0003-2697(87)90612-9

Seipke, R. F., Kaltenpoth, M., and Hutchings, M. I. (2012). *Streptomyces* as symbionts: an emerging and widespread theme? *FEMS Microbiol. Rev.* 36, 862–876. doi: 10.1111/j.1574-6976.2011.00313.x

Smith, S. E., and Smith, F. A. (2011). Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. *Annu. Rev. Plant Biol.* 62, 227–250. doi: 10.1146/annurev-arplant-042110-103846

Sun, J. H., Kelemen, G. H., Fernandez-Abalos, J. M., and Bibb, M. J. (1999). Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces* coelicolor A3(2). *Microbiology* 145, 2221–2227.

Tokala, R. K., Strap, J. L., Jung, C. M., Crawford, D. L., Salove, M. H., Deobald, L. A., et al. (2002). Novel plant-microbe rhizosphere interaction involving *Streptomyces* lydicus WYEC108 and the pea plant (*Pisum sativum*). *Appl. Environ. Microbiol.* 68, 2161–2171. doi: 10.1128/AEM.68.5.2161-2171.2002

van Veen, J. A., van Overbeek, L. S., and van Elsas, J. D. (1997). Fate and activity of microorganisms introduced into soil. *Microbiol. Mol. Biol. Rev.* 61, 121–135.

Watve, M., Tickoo, R., Jog, M., and Bhole, B. (2001). How many antibiotics are produced by the genus *Streptomyces*? *Arch. Microbiol.* 176, 386–390. doi: 10.1007/s002030100345

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16s Ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697–703.

Weyens, N., Boulet, J., Adriaensen, D., Timmermans, J.-P., Prinsen, E., Van Oevelen, S., et al. (2012). Contrasting colonization and plant growth promoting capacity between wild type and a gfp-derative of the endophyte *Pseudomonas putida* W619 in hybrid poplar. *Plant Soil* 356, 217–230. doi: 10.1007/s11104-011-0831-x

Yuan, W. M., and Crawford, D. L. (1995). Characterization of *Streptomyces lydicus* WYEC108 as a potential biocontrol agent against fungal root and seed rots. *Appl. Environ. Microbiol.* 61, 3119–3128.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 13 November 2014; accepted: 08 January 2015; published online: 06 February 2015.

Citation: Bonaldi M, Chen X, Kunova A, Pizzatti C, Saracchi M and Cortesi P (2015) Colonization of lettuce rhizosphere and roots by tagged Streptomyces. Front. Microbiol. ***6****:25. doi: 10.3389/fmicb.2015.00025*

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Microbiology.

Biological Control of Fungal and Bacterial Plant Pathogens
IOBC-WPRS Bulletin
In press

Labeling of promising biological control streptomycetes with EGFP

Xiaoyulong Chen, Andrea Kunova, Maria Bonaldi , Cristina Pizzatti, Marco Saracchi, Paola Sardi and Paolo Cortesi
University of Milan, Department of Food, Environmental and Nutritional Science, Via Celoria 2, 20133 Milano, Italy, email: paolo.cortesi@unimi.it

Abstract: Soil-borne fungal pathogens cause serious damage to horticultural crops. One of the most serious is *Sclerotinia sclerotiorum*, whose management relies mainly on chemicals, and more recently on use of biological control agents (BCA). Modern and sustainable disease management strategies for short cycle hort-crops should shift from chemicals to known or new BCA. With the objective to broaden the number of BCA and to get a new insight into the mode of action of streptomycetes able to reduce *S. sclerotiorum* severity, we have applied an EGFP (enhanced green fluorescence protein) approach to study *Streptomyces*-mediated biological control. The EGFP gene was integrated through conjugation into five strains that had previously shown strong *in vitro* antagonistic activity. The ex-conjugates were selected based on their resistance to apramycin. Conjugation efficiencies varied among strains from 5.81×10^{-8} to 4.64×10^{-5} ex-conjugates/recipient cell. The ex-conjugates fluoresced when observed by fluorescence microscopy, and the presence of the EGFP gene was additionally confirmed by PCR. The influence of EGFP transformation on the growth rate and the antagonistic activity against *S. sclerotiorum in vitro* was evaluated. None of the characters were significantly altered for the ex-conjugates, except for EGFP-FT05W strain, which showed lower antagonistic activity. The EGFP transformed *Streptomyces* strains will be used to study plant-pathogen-microbe interactions, and will contribute to further understanding of biological control mechanisms of plant soil-borne fungal pathogens.

Key words: *Streptomyces*, enhanced green fluorescence protein, biological control, fitness

Introduction

Streptomyces spp., belonging to *Actinomycetes*, is an important genus of gram-positive, filamentous bacteria found ubiquitously in soil. Streptomycetes have been shown to suppress plant diseases and could be used as biological control agents in various hosts (Emmert & Handelsman, 1999; Singh *et al.*, 1999; Paulitz & Belanger, 2001; Minuto *et al.*, 2006). Colonization of the host plant by beneficial microbes plays a crucial role in biological control (Haas & Defago, 2005; Naik *et al.*, 2009). The scanning electron microscopy techniques have been used to determine the colonization of Streptomycetes in plants (Sardi *et al.*, 1992; Basil *et al.*, 2004). Besides scanning electron microscopy, the innovation and application of auto fluorescent proteins, such as the green fluorescent protein (GFP), originally found in the jellyfish *Aequorea victoria*, have been developed as novel markers for studying plant-microbe interactions, including bacteria colonization and biocontrol mechanisms (Bloemberg, 2007).

GFP emits fluorescence directly when excited by UV or short-wave blue light, eliminating thus the use of exogenous cofactors or substrates for its detection. However, the utilization of the wild-type GFP in streptomycetes often failed most probably because of different codon usage (Leskiw *et al.*, 1991; Sun *et al.*, 1999). Compared to wild-type GFP, the

enhanced green fluorescent protein (EGFP) gene is a modified version with a codon usage that corresponds well to that found in many GC rich streptomycete genes. The EGFP, originally developed for expression in mammalian cells, was adopted to report spatial and temporal gene expression in *S. coelicolor* (Sun et al., 1999). Recently, its use in streptomycetes to report gene expression and to determine protein dynamic localization, such as their roles in spore germination and mycelium formation, is rapidly increasing (Dedrick *et al.*, 2009; Heichlinger *et al.*, 2011; Jyothikumar *et al.*, 2012; Willemse *et al.*, 2012).

Here, five *Streptomyces* strains showing biocontrol potential against *S. sclerotiorum* have been labeled with EGFP to study their localization and interactions in/with the host plant, and to gain understanding about root and rhizosphere colonization which are crucial for their biocontrol activity against plant soil-borne fungal pathogens.

Material and methods

Strains and plasmids used

The *Streptomyces* strains used in this study were maintained in the Plant Pathology Laboratory, Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, and selected previously as potential biocontrol agents against plant soil-borne fungal pathogens (Sardi *et al.*, 1992; Petrolini *et al.*, 1996; Bonaldi *et al.*, 2011; Bonaldi *et al.*, 2014). For conjugation with selected *Streptomyces* spp., a methylation deficient strain *E.coli* ET12567 (harboring the helper plasmid pUZ8002), was kindly provided by prof. Flavia Marinelli, University of Insubria, Italy, and was used as the donor strain. The plasmid pIJ8641, obtained from prof. Mervyn Bibb, John Innes Center, UK, was maintained in *E.coli* strain DH5α. It carries the EGFP gene under the constitutive ErmE promoter, an apramycin resistance marker (*aac*(*3*)*IV*), an *oriT/*RK2 region, and a lambda phage chromosomal integration sequence (IntC31)(Sun *et al.*, 1999).

Conjugation

The plasmid pIJ8641 was extracted from *E. coli* DH5α by plasmid DNA minipreparation method (Birnboim & Doly, 1979) and transformed into the donor strain *E.coli* ET12567 (pUZ8002) by rubidium chloride method (Hanahan, 1983). Subsequently, it was conjugated into recipient *Streptomyces* strains as previously described (Kieser *et al.*, 2000). The concentration of the *E. coli* donor strain ET12567 containing plasmid pIJ8641 was adjusted to 10^7 CFU/ml prior to conjugation. The conjugation efficiency was calculated as the number of ex-conjugant colonies divided by the number of recipient spores. The strain *S. coelicolor* A3 (2) was used as a reference strain. The ex-conjugants were selected on the soya flour mannitol agar medium (SFM) (20g/L organic soya flour, 20g/L Mannitol Difco, and 20g/L agar) added with $MgCl_2$ (10mM) and 50 mg/L of apramycin.

Genomic DNA extraction and PCR amplification

The genomic DNA of ex-conjugants and wild-type *Streptomyces* strains was extracted by CTAB method (Kieser *et al.*, 2000), with an additional step to remove RNA in excess before adding isopropanol. The amplification of 16S rDNA fragment (expected size 1500 bp) was used to confirm the presence of DNA in all samples, using PCR primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and fD2 (5'-ACGGCTACCTTGTTACGACTT-3') (Weisburg *et al.*, 1991), and the thermal cycling conditions: an initial denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 92°C for 45 s, annealing at 56°C for 30 s and extension at 72°C for 2 min, followed by a final extension at 72°C for 5 min. The PCR

primers rEGFP-N (5'-CTGGTCGAGCTGGACGGCGACG-3') and rEGFP-C (5'-CACGAACTCCAGCAGGACCA TG-3') were designed to amplify the EGFP gene fragment (expected fragment size 700 bp). The thermal cycling conditions used were: an initial denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 92°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 2 min, followed by a final extension at 72°C for 2 min. The DNA amplification was carried out using PCR thermal cycler (BioRad, Italy), performed in a total volume of 25 µl containing 30-50 ng DNA, 0.25 µM each primer, 1 U/µl Go *Taq* DNA polymerase (Promega), 5x Go Taq buffer (Promega), and 0.2 mM of each dNTP. The PCR products were visualized by electrophoresis in 1% (w/v) agarose gel. The gel containing ethidium bromide (10 µg/ml) was viewed under transilluminator to confirm the expected size of the product.

Observation of protein expression

The ex-conjugants and wild-type *Streptomyces* strains were grown in liquid Czepek broth (CZY) (35 g/L Czapek-Dox Broth Difco, 2 g/L Yeast Extract Difco) for three days and subsequently observed by fluorescence microcopy, Olympus BX51, with the FITC filter set, with 467-498 nm excitation length and 513-556 nm emission length.

Antibiosis test

The antagonistic activity of ex-conjugates and wild-type *Streptomyces* strains against *S. sclerotiorum* was performed by the dual culture assay on CZY medium. Ten µl of spore suspension (10^7 CFU/ml) of *Streptomyces* were inoculated on a 40mm line two days prior to *S. sclerotiorum* inoculation. An agar-mycelium plug (5 mm diameter), obtained from the edge of an actively growing colony of *S. sclerotiorum* grown on Malt Extract Agar (MEA) (20 g/L Malt Extract, Difco and 15 g/L agar) was placed upside down in each dish at 25 mm distance from the growing Streptomyces colony, and the plates were incubated for 72 hours at 24^{o}C. Plate inoculated only with *S. sclerotiorum* plug was used as control. The experiment was repeated twice in three replicates. The percentage of inhibition (PI) of *S. sclerotiorum* radial growth was calculated using the formula: PI (%) = $C\text{-}R/C \times 100$, where *C* represents the distance from the inoculation point to the colony margin on the control plate, and *R* indicates the distance of fungal radial growth from the inoculation point to the colony margin on the *Streptomyces*-inoculated plate.

Growth rate determination

Twenty µl of *Streptomyces* spore suspension (10^7 CFU/ml) were tranfered into a falcon tube containing 20 ml of CZY, and incubated at 30 ^{o}C, with 200 rpm constant shaking. Daily growth measurments were done as follows; two ml of liquid culture were put into pre-weighted eppendorf tube and spined at 10000 rpm for 10 minutes. The supernatant was removed and the pellet was dried overnight at 70^{o}C in the oven. The eppendorf was weighted when the pellet was completely dry, and the difference between the two weight measurements corresponded to the dry weight of streptomycetes biomass. The measurements were done in four replicates.

Results

Conjugation efficiency

Six strains including *S. coelicolor* A3 (2) were transformed with pIJ8641 harboring the EGFP gene under the constitutive promoter. Conjugation efficiency varied among strains. Four

strains, CX14W, SW06W, CX16W and FT05W showed conjugation efficiency similar to *S. coelicolor* A3(2), while one strain, ZEA17I, demonstrated significantly lower conjugation efficiency than the reference strain (Table 1).

Table1. Conjugation efficiencies of five *Streptomyces* spp. strains and *Streptomyces coelicolor* with the pIJ8641 plasmid.

Strain	***Donor strain (CFU/ml)***	***Recipient strain (CFU/ml)***	***Conjugation efficiency (CFU/recipient strain)***
CX14W	10^7	10^8	4.64×10^{-5}
SW06W	10^7	10^8	3.13×10^{-5}
CX16W	10^7	10^{10}	6.88×10^{-6}
FT05W	10^7	10^9	1.60×10^{-6}
ZEA17I	10^7	10^9	5.81×10^{-8}
***S.coelicolor* A3(2)**	10^7	10^8	9.10×10^{-6}

PCR detection of the EGFP-transgene

Genomic DNA of both ex-conjugants and wild-type S*treptomyces* strains was extracted, and the PCR amplification was performed to verify the presence of the EGFP fragment to confirm successful transformation. 16S rDNA fragment was amplified as a control of DNA extraction (Figure1). The 16S rDNA fragment was amplified in all samples, while the EGFP gene fragment was only detected in transformed strains.

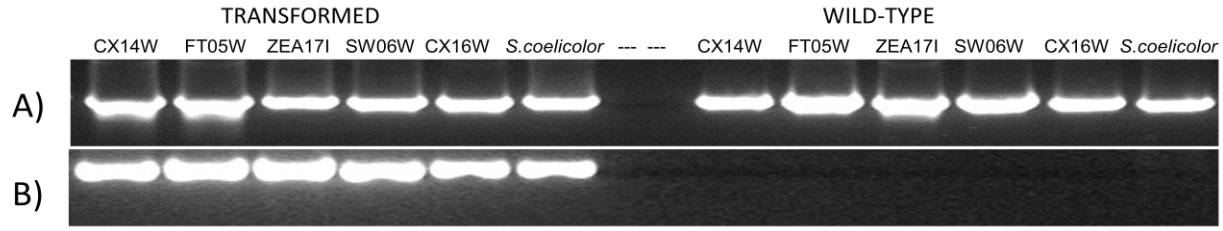


Figure 1. PCR amplification of EGFP fragment. A) PCR to detect 16S rDNA fragment. B) PCR to detect EGFP gene fragment. --- represents H_2O used as negative control.

Microscopic observation of EGFP expression

The expression of EGFP in transformed strains was observed by fluorescence microscopy, and all transformed strains fluoresced. The transformed strain showed consistent and strong green fluorescence upon exposition to fluorescent light (Figure 2b), while it's corresponding wild-type strain did not fluoresced (Figure 2d).

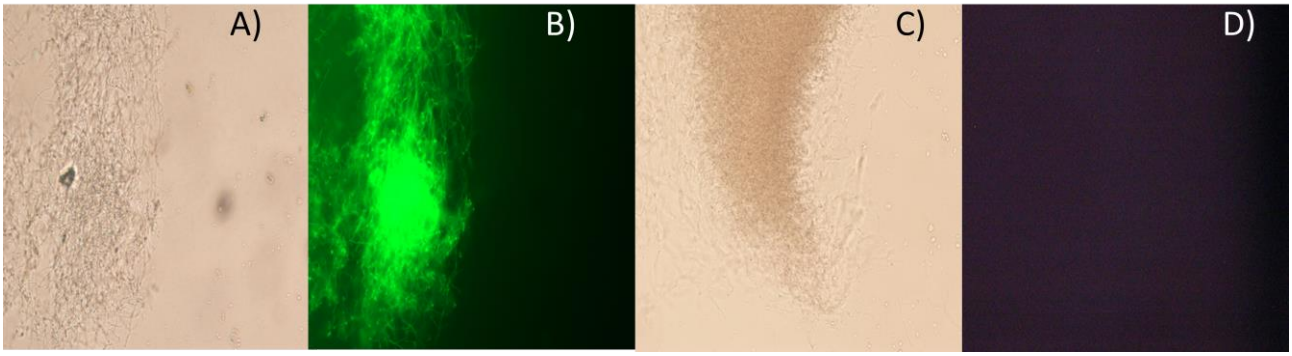


Figure 2. EGFP expression in *Streptomyces* spp. strain FT05W. A) The transformed strain observed under white light, and B) shortwave blue light, C) the wild-type strain observed under white light, and D) shortwave blue light.

Antibiosis test

The antagonistic activity of wild-type strains against *S. sclerotiorum* ranged from 66 to 81%. After conjugation, the EGFP-transformed strains inhibited *S. sclerotiorum* radial growth to similar extent as the corresponding wild-type strains, except for EGFP-FT05W strain, which showed approximately 33% lower inhibition than the FT05W wild-type strain (Figure 3).

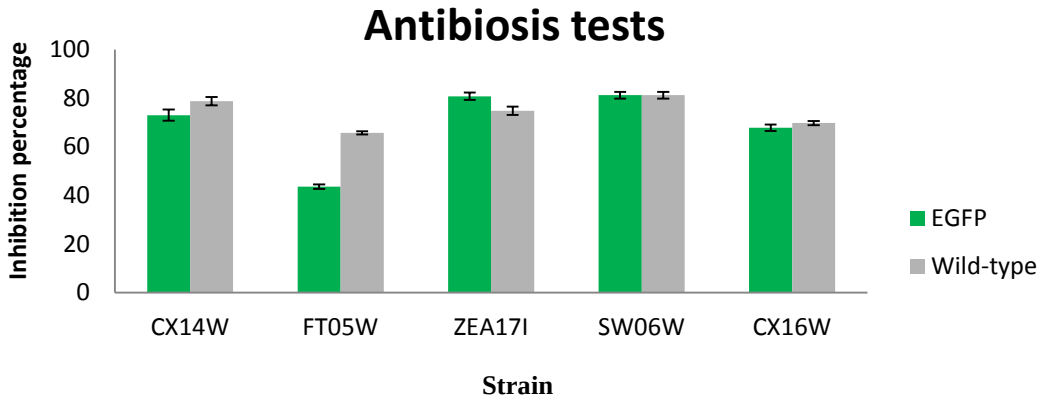


Figure 3: Inhibition of *Sclerotinia sclerotiorum* radial growth by EGFP- or wild-type *Streptomyces* spp. The error bars represent the standard error.

Growth rate determination

All the EGFP transformed strains showed similar growth rate to their corresponding wild-type strains. For brevity, the growth curve of EGFP-CX16W was similar to its wild-type strain (Figure 4a), and the same was observed for EGFP-FT05W, which showed lower antagonistic activity to *S. sclerotiorum* (Figure 4b).

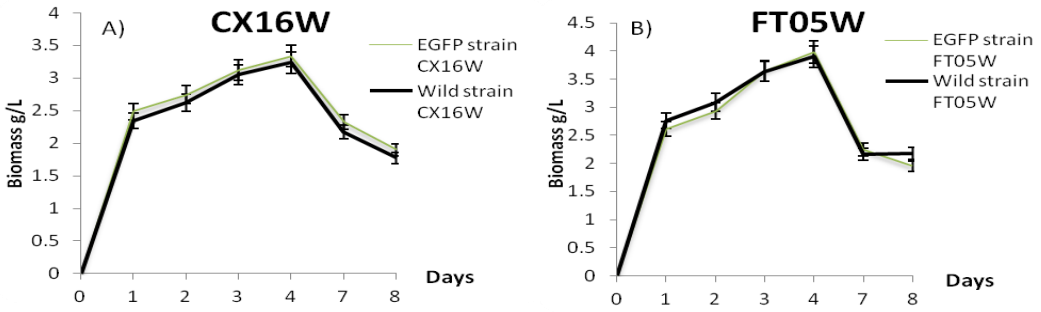


Figure 4. Growth rate (biomass) of *Streptomyces* spp. before and after transformation with EGFP. A) Strain FT05W. B) Strain CX16W. The error bars represent the standard error.

Disscussion

The EGFP construct (pIJ8641) was successfully conjugated into five target *Streptomyces* strains, with variable conjugation efficiency in different strains. The concentration of the donor strain was adjusted to 10^7 CFU/ml, as described in literature. In case of the recipient strains, variable spore concentrations were used (Paranthaman & Dharmalingam, 2003; Luzhetskyy *et al.*, 2006; Galm *et al.*, 2008), however, the minimum concentration that obtained successful ex-conjugates in our case was 10^8 CFU/ml. The presence of the EGFP gene in all transformed *Streptomyces* strains was confirmed by molecular means and by microscopic observation using fluorescence microscopy. The transformed strains showed consistent and strong green fluorescence upon exposition to fluorescent light. Therefore, the conjugation with the methylation defective *E. coli* ET12567 is an efficient method for transformation of diverse *Streptomyces* spp. and the pIJ8641 can be successfully used for visualization of transformed strains.

Transformation of a reporter gene is sometimes accompanied by changes in fitness or properties important for biocontrol of the selected strain. Indeed, three of five GFP-transformed *Metschnikowia pulcherrima* transformants lost antagonistic activity against *Botrytis cinerea*, while the activity of the remaining two transformants was indistinguishable from the wild-type strain (Nigro *et al.*, 1999). Similarly, after the transformation of *Clonostachys rosea* with β-glucuronidase (GUS) and GFP reporter genes, most transformants maintained similar biocontrol efficiency against *Fusarium culmorum* to the wild-type strain, however, one transformant showed decreased control efficiency (Lubeck *et al.*, 2002).

The *Streptomyces* strains used in our study were selected on the basis of their strong antagonism against *S. sclerotiorum in vitro* (Bonaldi *et al.*, 2014). Prior to studying and visualizing the interactions of bioactive *Streptomyces* with the host plant and the pathogen by fluorescence microscopy, the fitness of the EGFP- tagged strain was compared to that of wild-type. The antibiosis test against *S.sclerotiorum*, one of the most important soil-borne pathogens causing serious damage in horticultural plants, was done to see if the biocontrol potential of transformed strains was preserved after transformation. In four of five strains, there were no significant differences between their antagonistic activities against *S.sclerotiorum in vitro* before and after transformation. Instead, the *Streptomyces* FT05W strain showed significantly lower antagonism after transformation with EGFP construct.

Moreover, the effect of EGFP transformation on the growth rate, determined by the biomass dry weight was studied. All the five transformed strains showed similar growth rate to the corresponding wild-type ones, including the *Streptomyces* EGFP-FT05W strain, in which we observed lower antagonistic activity against *S.sclerotiorum.*

Before applying these EGFP labeled strains to study their colonization in plant tissues and biocontrol, further experiments will be performed to assess other characteristics of fitness after transformation, such as spore and secondary metabolites production, which may be involved in biocontrol mechanisms of *Streptomyces* against plant soil-borne pathogens.

Acknowledgement

We would like to thank prof. Mervyn Bibb, John Innes Center, UK, for providing us the pIJ8641 plasmid, and prof. Flavia Marinelli, University of Insubria, Italy, for the donor strain *E. coli* ET12567 used for conjugation.

References

Basil, A.J., Strap, J.L., Knotek-Smith, H.M., Crawford, D.L., 2004. Studies on the microbial populations of the rhizosphere of big sagebrush (Artemisia tridentata). J Ind Microbiol Biot 31, 278-288.

Birnboim, H.C., Doly, J., 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7, 1513-1523.

Bloemberg, G.V., 2007. Microscopic analysis of plant-bacterium interactions using auto fluorescent proteins. Eur J Plant Pathol 119, 301-309.

Bonaldi, M., kunova, A., Saracchi, M., Sardi, P., Cortesi, P., 2014. Strptomycetes as Biological Control Agents against Basal Drop. Acta Horticulturae.

Bonaldi, M., Kunova, A., Sardi, P., Cortesi, P., Saracchi, M., 2011. Streptomycetes as possible biocontrol agents against soil-borne pathogens. J Plant Pathol. Edizioni ETS, Pisa, Italy, pp. S4. 26-S24. 27.

Dedrick, R.M., Wildschutte, H., McCormick, J.R., 2009. Genetic Interactions of smc, ftsK, and parB Genes in Streptomyces coelicolor and Their Developmental Genome Segregation Phenotypes. J Bacteriol 191, 320-332.

Emmert, E.A.B., Handelsman, J., 1999. Biocontrol of plant disease: a (Gram-) positive perspective. Fems Microbiol Lett 171, 1-9.

Galm, U., Wang, L.Y., Wendt-Pienkowski, E., Yang, R.Y., Liu, W., Tao, M.F., Coughlin, J.M., Shen, B., 2008. In vivo manipulation of the bleomycin biosynthetic gene cluster in Streptomyces verticillus ATCC15003 revealing new insights into its biosynthetic pathway. J Biol Chem 283, 28236-28245.

Haas, D., Defago, G., 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 3, 307-319.

Hanahan, D., 1983. Studies on transformation of Escherichia coli with plasmids. J Mol Biol 166, 557-580.

Heichlinger, A., Ammelburg, M., Kleinschnitz, E.M., Latus, A., Maldener, I., Flardh, K., Wohlleben, W., Muth, G., 2011. The MreB-Like Protein Mbl of Streptomyces coelicolor A3(2) Depends on MreB for Proper Localization and Contributes to Spore Wall Synthesis. J Bacteriol 193, 1533-1542.

Jyothikumar, V., Klanbut, K., Tiong, J., Roxburgh, J.S., Hunter, I.S., Smith, T.K., Herron, P.R., 2012. Cardiolipin synthase is required for Streptomyces coelicolor morphogenesis. Mol Microbiol 84, 181-197.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., Hopwood, D.A., 2000. Practical Streptomyces Genetics. The John Innes Foundation, Norwish, UK.

Leskiw, B.K., Lawlor, E.J., Fernandezabalos, J.M., Chater, K.F., 1991. Tta Codons in Some Genes Prevent Their Expression in a Class of Developmental, Antibiotic-Negative, Streptomyces Mutants. P Natl Acad Sci USA 88, 2461-2465.

Lubeck, M., Knudsen, I.M.B., Jensen, B., Thrane, U., Janvier, C., Jensen, D.F., 2002. GUS and GFP transformation of the biocontrol strain Clonostachys rosea IK726 and the use of these marker genes in ecological studies. Mycol Res 106, 815-826.

Luzhetskyy, A., Fedoryshyn, M., Gromyko, O., Ostash, B., Rebets, Y., Bechthold, A., Fedorenko, V., 2006. IncP Plasmids are most effective in mediating conjugation between Escherichia coli and Streptomycetes. Russ J Genet 42, 476-481.

Minuto, A., Spadaro, D., Garibaldi, A., Gullino, M.L., 2006. Control of soilborne pathogens of tomato using a commercial formulation of Streptomyces griseoviridis and solarization. Crop Prot 25, 468-475.

Naik, B.S., Shashikala, J., Krishnamurthy, Y.L., 2009. Study on the diversity of endophytic communities from rice (Oryza sativa L.) and their antagonistic activities in vitro. Microbiol Res 164, 290-296.

Nigro, F., Sialer, M.M.F., Gallitelli, D., 1999. Transformation of Metschnikowia pulcherrima 320, biocontrol agent of storage rot, with the green fluorescent protein gene. J Plant Pathol 81, 205-208.

Paranthaman, S., Dharmalingam, K., 2003. Intergeneric conjugation in Streptomyces peucetius and Streptomyces sp strain C5: Chromosomal integration and expression of recombinant plasmids carrying the chiC gene. Appl Environ Microb 69, 84-91.

Paulitz, T.C., Belanger, R.R., 2001. Biological control in greenhouse systems. Annu Rev Phytopathol 39, 103-133.

Petrolini, B., Quaroni, S., SARACCHI, M., Sardi, P., 1996. Studies on the Streptomycete population inhabiting plant roots. Actinomycetes 7, pp. 66-78.

Sardi, P., Saracchi, M., Quaroni, S., Petrolini, B., Borgonovi, G.E., Merli, S., 1992. Isolation of Endophytic Streptomyces Strains from Surface-Sterilized Roots. Appl Environ Microb 58, 2691-2693.

Singh, P.P., Shin, Y.C., Park, C.S., Chung, Y.R., 1999. Biological control of Fusarium wilt of cucumber by chitinolytic bacteria. Phytopathology 89, 92-99.

Sun, J.H., Kelemen, G.H., Fernandez-Abalos, J.M., Bibb, M.J., 1999. Green fluorescent protein as a reporter for spatial and temporal gene expression in Streptomyces coelicolor A3(2). Microbiol-Uk 145, 2221-2227.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16s Ribosomal DNA Amplification for Phylogenetic Study. J Bacteriol 173, 697-703.

Willemse, J., Ruban-Osmialowska, B., Widdick, D., Celler, K., Hutchings, M.I., van Wezel, G.P., Palmer, T., 2012. Dynamic Localization of Tat Protein Transport Machinery Components in Streptomyces coelicolor. J Bacteriol 194, 6272-6281.

The abstract has been accepted by the *Frontiers* research topic: Microbiome interplay and control. The full manuscript is under preparation to be submitted.

frontiers

About | Submit | Journals | Research Topics

Search for articles, people, events and more.

Research Topic

Microbiome interplay and control

Submit an abstract | Submit a manuscript

About this Research Topic

In complex systems, such as our body or a plant, the host is living together with thousands of microbes, which support the entire system in function and health. The stability of a microbiome is influenced by environmental changes, introduction of microbes and microbial communities, or other factors. As learned in the past, microbial diversity is the key and low-diverse microbiomes often mirror out-of-control situations or disease.

It is now our task to understand the molecular principles behind the complex interaction of microbes in, on and around us in order to optimize and control the function of the microbial community – by changing the environment or the addition of the right microorganisms.

This research topic focuses on studies (including e.g. original research, perspectives, minireviews, commentaries and opinion papers) that investigate and discuss:

1) The role of the microbiome for the host/environmental system

2) The exchange and change of microbes and microbial communities (interplay)

3) The influence of external factors toward the stability of a microbiome

4) Methods, possibilities and approaches to change and control a system's microbiome (e.g. in human or plant disease)

5) Experimental systems and approaches in microbiome research

Selection of *Streptomyces* against soil borne pathogens by a standardized dual culture assay and evaluation of their effects on plant growth

Maria Bonaldi, Marco Saracchi, Andrea Kunova, Xiaoyulong Chen, Cristina Pizzatti and Paolo Cortesi[1]

Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, via Giovanni Celoria, 2 20133 Milano (Italy)

E-mail addresses: maria.bonaldi@unimi.it (M. Bonaldi), marco.saracchi@unimi.it (M. Saracchi), andrea.kunova@unimi.it (A. Kunova), chen.xiaoyulong@unimi.it (X. Chen), cristina.pizzatti@unimi.it (C. Pizzatti), paolo.cortesi@unimi.it (P. Cortesi)

[1] paolo.cortesi@unimi.it (P. Cortesi) +390250316771 fax +390250316748

Award No.1

International Organisation for Biological and Integrated Control of Noxious Animals and Plants (IOBC)
West Palaearctic Regional Section (WPRS)
Organisation Internationale de Lutte Biologique et Intégrée contre les Animaux et les Plantes Nuisibles (OILB)
Section Régionale Ouest Paléarctique (SROP)

Biocontrol of Plant Diseases: "From the field to the laboratory and back again"

The XIII Meeting of the IOBC/WPRS Working Group
Biological control of fungal and bacterial plant pathogens

has awarded

Xiaoyulong Chen

as

WINNER OF A STUDENT POSTER AWARD

Uppsala, 15-18 June, 2014

SLU, Swedish University of Agricultural Sciences

Award No.2

XXI Convegno Nazionale Società Italiana di Patologia Vegetale (SIPaV)

The XXI National Congress of Italian Phytopathological Society (SIPaV)

CERTIFICATION

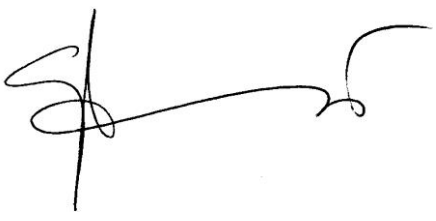
Here to certify that *Mr. Xiaoyulong Chen,* born in 20/06/1988, was one of the representative speakers of the winners of the competitive "*Giovani in Formazione*" Grant from SIPaV to offer an oral presentation at the *XXI National Congress of the Italian Phytopathological Society* (SIPaV).

Torino, 21-22-23 Settembre 2015

Turin, Italy

From the president of SIPaV

(Prof. G. Vannacci)



Congress Site
Torino Incontra Congress Center
Via Nino Costa 8
Turin, Italy