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PhD thesis

Streptomycetes as Biological Control Agents and Plant Growth-Promoting Bacteria

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Non basta guardare,

occorre guardare con occhi che vogliono vedere,

che credono in quello che vedono

Galileo Galilei

Abstract

Developing no-chemical strategies for the control of soil borne pathogens is one of the major issues for the cultivation of leafy vegetables. The application of Biological Control Agents (BCAs) represents a valuable approach and nowadays some biocontrol products are available on the market for greenhouse and field applications. However, these products often show lack of consistency and variable results mainly due to the poor knowledge about their biology and modes of applications and how the agroecosystem components modulate their efficacy.

Streptomycetes are soil inhabitants and have an important ecological role in the turn-over of organic matter; they can also establish beneficial relationships with plant roots enhancing host growth and protection against pathogens through the production of bioactive compounds, lytic enzymes, phytohormones and siderophores.

This PhD project aimed to study streptomycetes as BCA and Plant Growth Promoting Bacteria for their use to manage soil borne fungal epidemics in horticulture.

A collection of 200 endophytic streptomycete strains isolated from roots was used in this work. To be able to compare the activity of every strain against the pathogens, the dual culture assay was optimized for some representative fungal pathogens based on mycelium radial growth rate *in vitro*. Subsequently, the optimized method was applied to screen the collection. Some strains showed strong inhibitory activity, but it was specific for one target pathogen and in few cases comprised more than one pathogen. Based on the promising results obtained from the *in vitro* assays for *Sclerotinia sclerotiorum*, further studies were focused on the activity of ten strains used for biological control of lettuce drop in different conditions. In particular, it was studied the effect of the application timing of the antagonist and the pathogen, and the amount of the streptomycete used to improve lettuce drop protection. The survival analysis applied to the data of the growth chamber experiments showed that when lettuce was sown one week after the growth substrate inoculation with the pathogen and antagonists, disease control improved, and *Streptomyces* spp. FT05W, SW06W and SW29W reduced the risk of disease incidence by 42%. On the contrary, no beneficial effect was observed when lettuce was sown the same day of the growth substrate inoculation. Streptomycetes spore concentration significantly influenced lettuce drop protection, but this effect was strain-dependent.

Based on these results we planned appropriate field experiment to confirm the results obtained, however, in the field we did not observed significant differences in lettuce protection. Therefore we speculated that moving from controlled to a more complex agroecosystem environment the streptomycete antagonistic activity could fade away probably due to unfavorable interaction in a more complex microflora.

Indole-3-acetic acid and the siderophore production were observed for *Streptomyces* spp. CVM02R and SW29W in *in vitro* assays, but in field experiments no significant PGP effect on lettuce was obtained at harvest assessing the head weights of plants.

The colonization of lettuce rhizosphere and root tissues was investigated using the EGFP labelled strain *Streptomyces* sp. ZEA17I. This strain showed both rhizospheric and endophytic competences, characters necessary for its successful use for biological control. In addition we showed that applying the strains as spore suspension in the growth substrate resulted in significantly higher roots and rhizosphere colonization than when delivered as seed coating.

In conclusion, the results obtained in this study showed that bacteria of the genus *Streptomycetes* appear valuable candidates for the biological control of soil borne fungal pathogens. However, the complex interactions among the host plant, the antagonist and the pathogen occurring in the agroecosystem are mostly unknown and could generate contradictory results for different environments. Therefore, we think that further studies on simplified models are necessary in order to understand the mechanism on which biological control is based, in order to improve streptomycete activity as BCA for the management of fungal soil borne epidemics.

Keywords: soil borne pathogens, *Sclerotinia sclerotiorum*, lettuce, streptomycetes, biological control, colonization.

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Introduction

1 Horticulture in Italy

1.1 Overview

In Italy, vegetable crops are grown throughout the country with a cultivation area of approximately 3.5 million ha, both in open field and under protection, and a total yield of 10 million t (Istat, 2013). The cultivation under protection, such as plastic, glass houses or tunnels, is the most important part of this agricultural sector; indeed, it covers 3 million ha and only 0.3 million ha crops are grown in open field (Istat, 2013).

The cultivation of certain species is concentrated in few regions due to the optimal growing conditions, mostly in southern Italy (Table 1.2.1 and Table 1.2.2). Moreover, because of the close linkage with the territory in which they are cultivated and processed in specific high quality products, some vegetables are protected with the denominations "Protected Designation of Origin" (PDO) and "Protected Geographical Indication" (PGI). Some of the most famous protected products are: tomato "Pomodorino di Pachino PGI" in Sicily, chicories "Insalata di Lusia PGI", "Radicchio Rosso di Chioggia PGI", "Radicchio Rosso di Treviso PGI" in Veneto, basil "Basilico Genovese PDO" in Liguria, etc (Mipaaf, 2014).

In Italy, the most yielded vegetable crop is tomato and Italy is the first country in Europe for its production with approximately 5 million t (Eurostat, 2013). It is cultivated both in open field and under protection (Figure 1.4.1). Apart from tomato, a wide range of crops are cultivated. They belong to different families: Solanaceae (tomato, eggplant, pepper and potato, Asteraceae (lettuce, chicory, endive and artichoke), Liliaceae (onion and garlic), Cucurbitaceae (zucchini, melon, watermelon and cucumber), Apiaceae (carrot, fennel), etc.

1.2 Salad crops

This research is focused on soil borne disease management of salads; therefore some remarks and data about these vegetable crops are reported.

With the term "salads" are intended the leafy vegetables belonging to Asteraceae family. They are: lettuce (*Lactuca sativa* L.), curly endive (*Cichorium endive* L. var. crispum), escarole endive (*Cichorium endive* L. var. latifolium) and chicory (*Cichorium intybus* L.). Different types of lettuce are cultivated: romaine, green leaf, iceberg, batavia, crisphead and butterhead.

Table 1.2.1 – Yield and cultivated area of the main vegetable crops grown in open field in Italy and region with the highest production with relative yield.

Vegetable crop	Cultivated area (ha)	Yield (1 000t)	Region and yield (1 000t)
Tomato(*)	91850	5132	Puglia (1687)
Melon	20228	4629	Sicily (164)
Fennel	16000	4230	Puglia (126)
Antichoke	33296	3443	Sicily (153)
Carrot and Parsnip	9000	341	Sicily (101)
Lettuce	13610	290	Puglia (92)
Zucchini	11815	285	Sicily (62)
Onion	9509	267	Emilia-Romagna (83)
Eggplant	8053	208	Campania (62)
Chicory	13248	200	Veneto (102)

* ISTAT, 2012

Table 1.2.2 – Yield and cultivated area of the main vegetable crops grown in greenhouse in Italy and region with the highest production with relative yield.

Vegetable crop	Cultivated Area (ha)	Yield (1 000t)	Region and yield (1 000t)
Tomato	572434	354	Sicily (197)
Zucchini	366993	162	Lazio (99)
Other vegetables	381821	129	Campania (89)
Lettuce	373231	127	Campania (53)
Melon	259739	96	Lombardy (27)
Pepper	206481	92	Sicily (48)
Eggplant	134922	73	Sicily (39)
Watermelon	146105	72	Campania (29)
Strawberry	245115	65	Campania (31)
Cucumber	57199	35	Veneto (14)

In Italy, salads are grown both in open field and under protection all the year round (Figure 1.4.2 and Figure 1.4.3). The cultivation area in open field is approximately 34000 ha: 40% for lettuce, 38% for chicory and 22% for endive (Table 1.2.3). Lettuce and endive are mainly grown in southern Italy, especially in Puglia, whereas chicory in northern Italy, in Veneto. Lettuce is the most yielded crop followed by chicory and endive for a total production of 660000 t and a value of 629 million € (Casati, 2014; Istat, 2013).

Salad vegetables are also grown under protection with an area of 76000 ha: 49% lettuce, 26%, endive and 25% chicory. Unlike the open field, only lettuce is cultivated in southern Italy, mainly in

Campania, whereas chicory and endive are grown in northern Italy, in Lombardy and in Veneto, respectively (Table 1.2.4) (Istat, 2013).

Table 1.2.3 – Yield and cultivated area of salad crops in open field in Italy.

Crop		**Yield (1 000t)**	**Cultivated area (ha)**
Chicory	North	128	9652
	Centre	24	1148
	South	48	2448
	Italy	**200**	**13248**
Endive	North	18	802
	Centre	23	922
	South	128	5766
	Italy	**169**	**7490**
Lettuce	North	75	2712
	Centre	22	1619
	South	193	9279
	Italy	**291**	**13610**

Table 1.2.4 – Yield and cultivated area of salad crops in greenhouse in Italy.

Crop		**Yield (1 000t)**	**Cultivated area (ha)**
Chicory	North	3.3	11405
	Centre	0.7	2224
	South	0.8	5034
	Italy	**4.8**	**18663**
Endive	North	2.5	10733
	Centre	1.4	7345
	South	0.4	1666
	Italy	**4.3**	**19744**
Lettuce	North	29	87177
	Centre	41	124136
	South	57	161918
	Italy	**127**	**37321**

1.3 Ready-to-eat salads

The “ready-to-eat” salads are vegetables prepared to be easily consumed. They are washed and cut leafy vegetables wrapped in plastic bags ready to be eaten.

They were born in the USA in 1960s and were introduced in Europe in 1970s, first in France, then in the United Kingdom, in Germany, in Switzerland and finally in Italy in 1990s (Zucconi, 2013).
The "packaged salads" have been a real innovation in the horticultural Italian scenario and, even if they are more expensive than the common vegetables, they obtained a positive outcome from the consumers, because they respond to the needs of people searching for healthy and fresh foods, but not having time to prepare them. Therefore, their practical and easy-to-use characteristics meet the needs of the modern society. In Italy, in the last 10 years, sales of ready-to-eat salads increased by 376% and only in the last few years they decreased probably due to the economic crisis. In 2012 the market value was 759 million € (Casati and Baldi, 2013).
The cultivation area for "ready-to-eat" vegetables is 6500 ha, half of it under protection, generally plastic tunnels (Figure 1.4.4), and the farms are located mainly in Lombardy and Campania (Ismea, 2011). The vegetable crops, often denoted as "baby leaf", are: lettuce (*L. sativa* mainly Batavia type), lamb's lettuce (*Valerianella locusta* L.), wild and cultivated rocket (*Diplotaxis tenuifolia* L. and *Eruca sativa* Mill., respectively) and spinach (*Spinacia oleracea* L.). They are marketed singularly or as mix of two or more leafy vegetables. In recent years even red chicory, crispy and escarole endive and lettuce entered the "ready-to-eat" mixed products, as cut leaves.

1.4 Features of horticultural farming

In the Italian scenario, the horticultural farms are specialized in the cultivation of the same specific vegetable species, or small group of species. For instance, the farms specialized in "ready-to-eat" crops only produce leafy vegetables, resulting in absence of crop rotation. Moreover, thanks to protection structures (tunnels and plastic houses) crops are cultivated also off-season and more growing cycles of the same crop are repeated all year round causing intensive use of soil, which turns into the commonly referred "old land syndrome".
From a phytopathology point of view, the consequence of monoculture is the development of destructive disease epidemics, especially of soil borne pathogen origin.

Figure 1.4.1 – Tomato (*Solanum lycopersicum* L.) greenhouse. Bra (Cn), July 2014.

Figure 1.4.2 – Chicory (Radicchio rosso di Treviso, *Cichorium intybus* L.) in open field. Quinto di Treviso (Tv), November 2013.

Figure 1.4.3 – Lettuce (*Lactuca sativa* L.) under plastic tunnel. Bra (Cn), July 2014.

Figure 1.4.4 – Lettuce (*Lactuca sativa* L.) for ready-to-eat market under plastic tunnel. Chiuduno (Bg), January 2013.

2 Soil borne pathogens

Soil borne pathogens belong to different groups: fungi (true fungi and oomycetes) and bacteria. In Italy, the most destructive are the soil borne fungi.

2.1 Soil borne pathogens: biology and epidemiology

2.1.1 Inoculum

Soil borne fungi are present in soil as mycelium and survival propagules (Table 2.1.1). The latter can persist in absence of hosts for long periods. Sclerotia are round-shaped hyphae aggregations with an outer black rind containing melanin, a compound which plays an important role in their protection from adverse conditions and microbial degradation (Saharan and Mehta, 2008). Chlamydospores - derived from terminal or intercalary cells of old hyphae in which nutrients are accumulated - are characterized by a thick resistant cell wall. These spores can survive in soil for many years, but probably also germinate and maintain their population by growing in a saprotrophic mode in the rhizosphere (Deacon, 2005). The chlamydospores of *Thielaviopsis basicola* Berk. and Br. are dark-colored and are produced on infected root tissues, giving name to the disease: "black root rot" (Papavizas and Lewis, 1970). Oospores are thick-walled sexual spores resistant to desiccation and can survive on organic substrate that supports saprophytic growth.

2.1.2 Invasion and colonization

When the environmental conditions are favorable, the survival structures are chemoattracted by root or seed exudates and stimulated to germinate and grow. The germ tube or zoospore can attach to the surface of the root, penetrate and infect the epidermal cells of root tips, secondary roots, and root hairs, or attack the emerging shoots and radicles of seedlings. Some fast growing pathogens, such as *Pythium* species, can attack seeds and embryos before they emerge (Martin and Loper, 1999). Almost all soil borne fungi are necrotrophic, meaning they kill host tissues with enzymes and toxins released from hyphae, therefore they do not require living cells to obtain nutrients. Mycelium can continue to colonize the root, internally or externally, or can invade other roots in close proximity. *Fusarium oxysporum* Schlechtend. and *Verticillium dahliae* Kleb. can penetrate through the endodermis into the vascular tissues and move up through the xylem to above-ground parts of the plant, hampering water flow. Once a root is infected, the pathogen can spread to adjacent roots. For instance, *Pythium* and *Phytophthora* species release motile swimming zoospores that can swim to the adjacent roots (Martin and Loper, 1999).

2.1.3 Host range

Soil borne fungi can attack a broad host range of vegetable crops and only few pathogens show host specificity. *F. oxysporum* consists of more than 120 *formae specialis*, each showing a characteristic pattern of virulence on different host species. *Rhizoctonia solani* (Cooke) Wint. (teleomorph = *Thanatephorus cucumeris* Frank (Donk)) shows different host specificity on the basis of anastomosis group (AG). Isolates of AG1 cause seed and hypocotyl rot and web blights of many plant species, isolates of AG2 affect crucifers and turfgrasses, isolates of AG3 affect mostly potato and some AGs are not pathogens (Agrios, 2005; Anderson, 1982; Garcia *et al.*, 2006).

2.1.4 Symptoms

Soil borne pathogens can cause seed decay, damping-off (both pre- and post-emergence) and move into the base of the stem, causing basal rot and vascular wilt. By killing root tips, root growth on that axis is stopped. By destroying fine feeder roots and root hairs, water uptake ability of the plant is hampered. Subsequently, plants show reduced size, stunting, withering and can also die.
Soil borne diseases are difficult to diagnose based on symptoms, because most of them occur below ground, and the above-ground symptoms are not specific and distinctive, often similar to those caused by abiotic factors such as drought, lack of nutrients, etc.

Table 2.1.1 – Main soil borne pathogens, survival propagules, host range and symptoms.

Pathogen	**Survival propagules**	**Host range**	**Symptom**
Fusarium oxysporum	chlamydospores	Depending on the *formae specialis*	Vascular wilt
Sclerotinia spp.	sclerotia	wide	Basal drop, Lettuce drop,
Pythium ultimum	oospores	wide	Damping-off, seed rot
Phytophthora spp.	oospores	wide	Damping-off
Rhizoctonia solani	sclerotia	Depending on anastomosis group	Damping-off
Thielaviopsis basicola	chlamydospores	wide	Black root rot
Verticillium dahliae	microsclerotia	wide	Vascular wilt

2.2 Soil borne diseases of lettuce and related salad crops

Numerous soil borne pathogens can attack salad plants all year round and often several pathogens are simultaneously present in different periods of the year. On salad crops, in addition to the known pathogens, others have been reported in Italy in the last 10 years as consequence of intensive growing cycles (Gilardi *et al.*, 2010; Gullino *et al.*, 2007)

In spring and summer, when temperatures are between 25-35°C, symptoms of wilt disease caused by *F. oxysporum* can be observed (Garibaldi *et al.*, 2002). Affected plants appear stunted; develop yellow leaves and brown streaks in the vascular system. On lettuce, the causal agent is *F. oxysporum* f.sp. *lactucae* (Garibaldi *et al.*, 2002). The pathogen is transmitted by infected seeds (Garibaldi *et al.*, 2004a). Three *F. oxysporum* f.sp. *lactucae* pathogenicity groups are known worldwide. Group 1 is highly pathogenic to lettuce cultivars of crisphead and red leaf types and less pathogenic to butterhead and green leaf type cultivars, group 2 is highly pathogenic to the butterhead type and less pathogenic to crisphead and leaf types group, group 3 is less pathogenic to all lettuce types compared to groups 1 and 2 (Yamauchi *et al.*, 2001). To date, only Group 1 has been reported in Italy (Garibaldi *et al.*, 2004b). *F. oxysporum* f.spp. *raphanis* and *conglutinans* have been reported on wild and cultivated rocket as well as on lamb's lettuce (Garibaldi *et al.*, 2003; Gilardi *et al.*, 2010). These two pathogens are also transmitted by infected seeds (Garibaldi *et al.*, 2004c). *Fusarium* wilt is known on endive, too (Garibaldi *et al.*, 2009).

In the past, seedling Verticillium wilt could be observed in lettuce fields when temperatures were between 20-25°C. However, nowadays the pathogen (*V. dahlie*) does not seem to be dangerous for lettuce crops in Italy (Garibaldi *et al.*, 2007). At the end of summer, *T. basicola* can attack lamb's lettuce roots (Garibaldi *et al.*, 2005a). The pathogen reduces seedling growth, and since infected root tissues turn brown, the final product cannot be commercialized. *R. solani* can infect seedlings when temperature ranges between 15-22°C. Symptoms of water-soaked zonate lesions on basal leaves can be observed. The disease known as bottom rot is known on lettuce and lamb's lettuce (Garibaldi *et al.*, 2006; Gilardi *et al.*, 2010). In spring and fall, *Sclerotinia* spp. can also be present.

2.3 Basal drop

The disease known as “drop” is caused by *Sclerotinia* spp. Basal drop and lettuce drop have been reported in all the world and all types of lettuce and salad crops can be affected by *Sclerotinia* spp. (Subbarao, 1998).

2.3.1 Causal agents

Lettuce drop is caused by two closely related species, *S. sclerotiorum* Lib (de Bary) and *S. minor* Jagger. The fungi are most frequently found in cool and moist regions (Purdy, 1979). The disease may be caused by both species, which can occur together in the same field (Abawi and Grogan, 1979). Both pathogens produce sclerotia as survival propagules (Figure 2.3.1). Sclerotia of *S.minor* are mostly circular (0.5 to 2 mm diameter) whereas *S. sclerotiorum* produces irregular and bigger sclerotia (2 to 20 x 3 to 70 mm) (Subbarao, 1998). While carpogenic germination and production of apothecia are extremely rare in *S. minor*, *S. sclerotiorum* can germinate carpogenically, producing one to several apothecia, cup-shaped and usually white, yellow, or a shade of brown (Subbarao, 1998). For both species host specificity is not known. *S. sclerotiorum* infects a wide range of host plants, including many economically important crop species (Boland and Hall, 1994; Saharan and Mehta, 2008), whereas *S. minor* infects fewer hosts than *S. sclerotiorum* (Melzer *et al.*, 1997).

2.3.2 Disease cycle and pathogenesis

The characteristics that separate the two species of *Sclerotinia* also significantly impact the mode of infection of lettuce plants (Figure 2.3.2). Sclerotia play the principal role in disease cycles as they produce inoculum and are the primary long-term survival structures remaining viable for up to 8 years in soil (Adams and Ayers, 1979).

Sclerotia of *S. minor* have a dormancy period before mycelia growth, and the length of the dormancy varies among isolates and is affected by different environmental factors (Abawi and Grogan, 1979). Once germinated, the mycelium can directly infect lettuce without the need of an exogenous energy source (Abawi and Grogan, 1979).

On the contrary, sclerotia of *S. sclerotiorum* germinate to form either a mycelium or apothecia. The type of germination is determined by environmental factors such as humidity and temperature. Among them, soil moisture is a critical factor for apothecia production; ascospore infections are often associated with irrigation events or periods of frequent and heavy rainfall (Bolton *et al.*, 2006).

Sclerotia that germinate myceliogenically produce hyphae that can directly attack plant tissues. Ascospores germinate on the surface of healthy tissues, but cannot infect the plant without an exogenous nutrient source and a film of water (Bolton *et al.*, 2006). Therefore, senescent or necrotic

tissues generally serve as the nutrient source to initiate ascospore germination and give rise to mycelial infection of the host plant. Hyphae resulting both from sclerotia and ascospores are hyaline, septate, branched and multinucleate.

The pathogen produces a wide array of degradative, lytic enzymes, such as endo- and exopectinases, cellulases, hemicellulases, and proteases, instrumental for host colonization (Lumsden, 1979). Moreover, upon the successful entry into the host, the pathogen produces oxalic acid, which lowers the pH of the host tissue and decreases host cell viability and therefore its ability to respond to pathogen colonization. Additionally, oxalic acid may also seize Ca^{2+} and the resulting calcium oxalate crystals may occlude xylem vessels, leading to wilt symptoms (Bolton *et al.*, 2006; Marciano *et al.*, 1983).

2.3.3 Symptoms

The common name of the disease on lettuce typifies the final symptoms observed on infected plants. Infected plants can collapse in less than two days, hence the name "drop". Lettuce drop occurs at two phenological stages. The first one occurs at the rosette stage or immediately after thinning (3 to 4 weeks after seedling emergence) on a very low percentage of plants. The second economically significant stage occurs at or near crop maturity (Figure 2.3.3). The initial symptom is a stressed appearance of lettuce head, light yellowing and wilting of the outermost leaves, followed by complete wilting. Layers of collapsed leaves lay flat on the soil surface, giving the plant a yellowish color (Figure 2.3.4). Subsequently, under moist conditions, the fungus produces a snowy white mycelium on infected plant parts. Black sclerotia are produced and are visible on the lower surface of the leaves touching the soil, around the crown, and throughout the taproot (Figure 2.3.5). The size depends on the fungal species and the temperature during their formation.

Figure 2.3.1 – Sclerotia of *Sclerotinia* sp. on a chicory leaf (left) and on lettuce (right).

Figure 2.3.2 – Disease cycles of *Sclerotinia sclerotiorum* and *S.minor* (Subbarao, 1998).

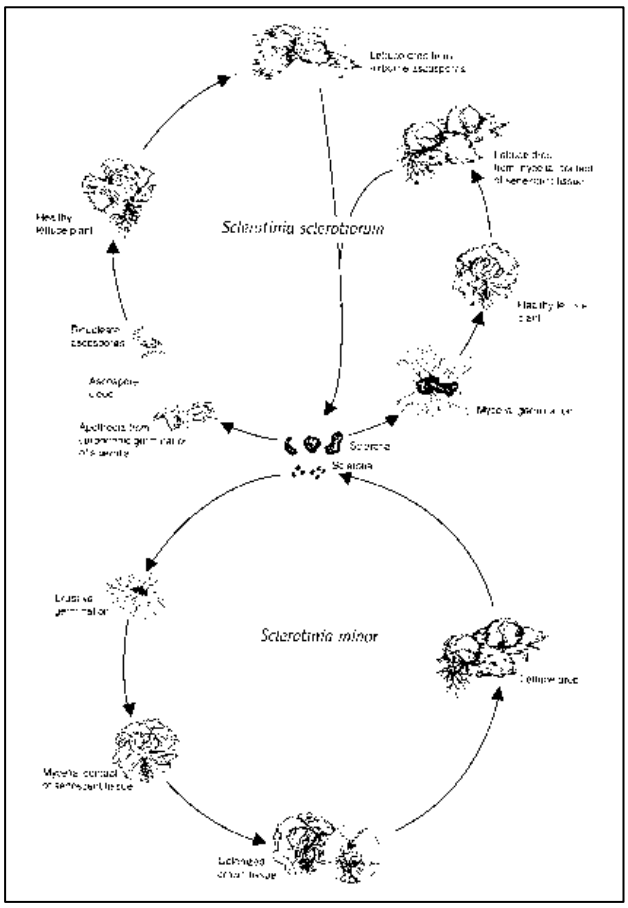


Figure 2.3.3 – Typical growth stages of crisphead lettuce (Subbarao, 1998).

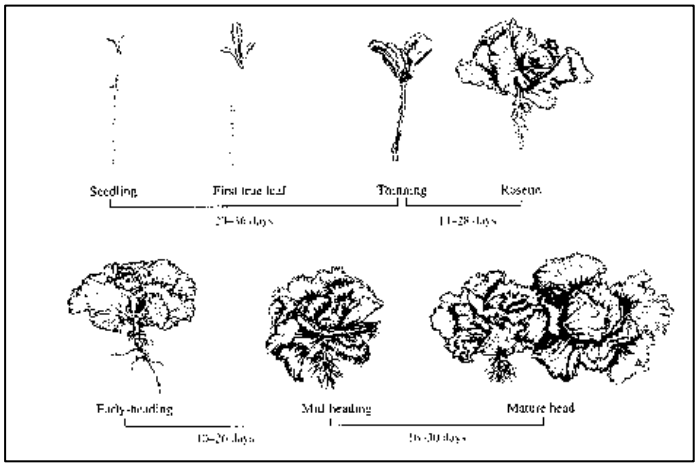


Figure 2.3.4 – Symptoms caused by *Sclerotinia* sp. on baby leaf lettuce.

Figure 2.3.5 - Symptoms caused by *Sclerotinia* sp. on chicory.

3 Soil borne diseases management

3.1 Integrated Pest/Disease Management

As imposed by Directive 2009/128/EC, by 2014 the management of plant diseases must follow the Integrated Pest/Disease Management (IPM/IDM) principles in all European countries. According to the Food and Agriculture Organization of the United Nations (FAO), IPM means the careful consideration of all available disease control techniques and subsequent integration of appropriate measures that discourage the development of pest/pathogen populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms.

Regarding the IDM of soil borne pathogens, it is of fundamental importance to lower the pathogen inoculum before plant sowing or transplanting. To reach this goal, different methods can be applied alone or in combination. They involve the application of fumigants, crop rotation, soil solarization, biofumigation, incorporation of organic amendments, and the introduction of biological control agents. The combination of methods for the control of pathogens is the major pillar of the IPM approach (Katan, 2014). However, combining different strategies is more than merely mixing two methods. The combination has to be optimal and the succession is also important (Katan and Gamliel, 2009). For instance, combining heating and fumigation resulted more effective in reducing germination of *Sclerotinia rolfsii* sclerotia than each treatment applied alone, moreover the sequence involving heating first, followed by fumigation, was significantly more effective than the opposite (Eshel *et al.*, 2000).

Lastly, in the case of salad crops, host resistance is considered an effective strategy for limiting the incidence of some pathogens; especially the seed transmitted pathogens such as different *formae speciales* of *Fusarium oxysporum*. For this purpose, a list was provided for the main Italian lettuce varieties resistant to *Fusarium oxysporum* f.sp. *lactucae* (Garibaldi *et al.*, 2004b).

3.2 Chemical strategies: fumigants

Soil fumigants have been used throughout the world for decades to control soil borne pests and pathogens prior to planting various food crops (Yates *et al.*, 2002).

From its first application in 1960s, methyl bromide (MBr) represented the most widely used chemical for the fumigation of soil and also of commodities, buildings and furniture (Gullino *et al.*, 2003). Thanks to the broad spectrum of activity, in agriculture MBr was applied to control fungi, nematodes and weeds. In 1995, Italy used more than 7600 metric tons of MBr, ranking first in

Europe and second in the world after the United States. The highest use of MBr was for fruit crops and vegetables, mainly tomatoes, strawberries, eggplants and ornamentals (Gullino *et al.*, 2003). Due to the discovery of the role of bromine atoms as major cause of ozone depletion in the stratosphere, MBr was added to the list of "ozone depleting substances" (ODS) and consequently several regulatory policies were adopted to reduce its consumption. According to the Montreal Protocol (1997), in the industrialized countries MBr was phased out until its complete withdraw from the market in 2005, whereas in the developing countries its use will be totally banned in 2015.
After the MBr withdraw other chemicals available on the market and were used in horticultural farming. However, to date the application of soil fumigants remains a critical point in the management of soil borne pathogens. The Regulation (EC) 1107/2009 concerning the placing of plant protection products on the market dramatically reduced the spectrum of available fumigants in the Italian and European scenario. Nowadays, chloropicrin as well as 1,3-dichloropropene are banned and only metham, including both –potassium and –sodium, and dazomet are authorized for soil applications prior to planting, limited to one application every three years on the same field. Moreover, the Directive 2009/128/EC on sustainable use of pesticide imposes the use of non-chemical methods for the plant diseases management thus, the use of fumigants is expected to encounter additional serious restrictions (Colla *et al.*, 2012).

3.3 Non-chemical strategies

3.3.1 Crop rotation

Crop rotation is one of the oldest tools to manage plant epidemics: thanks to the succession with nonhost crops, the pathogen cycle is stopped and its inoculum is reduced. Even though this strategy could provide a valid approach, it cannot be fully adopted in the Italian horticultural farms because they are highly specialized in the growing of specific vegetable species. And where this is not the case, soil borne fungi have often broad host range thus lowering the value of crop rotation.

3.3.2 Soil solarization

Soil solarization, or "solar heating of soil" as defined at that time, was first described in 1976 in an article reporting the results from laboratory and field studies against Verticillium wilt of eggplant and tomato in Israel (Katan *et al.*, 1976). The work represents the first of numerous studies carried out in the following years aimed to investigate the application of soil solarization in other countries and to increase the knowledge about its mechanisms of action (Katan *et al.*, 1987).
Fundamentally, during the soil solarization process, the soil surface is covered with a transparent plastic film to trap solar radiation. Films based on infrared (IR) blocking material are used (Chase *et*

al., 1999). Moreover, a different plastic formulation was tested using the addition of anti-drip (AD) components. This formulation prevents condensation of water droplets on the film surface, leading to a 30% increase in irradiation transmittance over regular film (Katan and Gamliel, 2009). During the solarization process, soil is kept wet in order to increase the sensitivity of the propagules to heat and to improve heat conduction. The process has mainly a physical effect on pathogen propagules which are killed by the high temperatures achieved during the process (Katan and Gamliel, 2009). However, the consequences of solarization are not limited only to the reduction of pathogen inoculum. Indeed, in solarized soil the population of beneficial organisms such as fluorescent pseudomonads, *Bacillus* spp., *Trichoderma* spp. and other competitors usually increased (Tjamos *et al.*, 2000). Moreover, solarized soils show long-term effects in plant protection because they are frequently less vulnerable to re-colonization (Katan and Gamliel, 2009). However, soil solarization has some limits. Due to the climate conditions, it can be applied only in certain regions and during certain periods of the year. Moreover, during the process the soil remains without crop for 3 to 6 weeks, or even more in the North, thus limiting its application in the Italian scenario of horticulture.

3.3.3 Biofumigation

Brassica species and other members of *Brassicacae* contain in their tissues significant quantities of the thioglucoside compounds known as glucosinolates (GSLs). GSLs are hydrolysed by the myrosinase enzyme (present endogenously in *Brassica* tissues) to release a range of hydrolysis products including oxazolidinethiones, nitriles, thiocyanates and various forms of volatile isothiocyanates (ITCs) (Gimsing and Kirkegaard, 2009; Halkier and Gershenzon, 2006). These hydrolysis products, in particular the ITCs, are known to have broad biocidal activity on insects, nematodes, fungi, bacteria and weeds (Brown and Morra, 1997; Kirkegaard and Sarwar, 1998; Motisi *et al.*, 2009; Sarwar *et al.*, 1998). "Biofumigation" is a term used to describe the suppression of soil borne pathogens and pests by *Brassica* species. It can be achieved by incorporating in soil fresh plant material (green manure), seed meals (a by-product of seed crushed for oil) or dried plant material treated to preserve isothiocyanate activity or by using brassica intercrops (Lazzeri *et al.*, 2004). Some experiments were carried out to study the possibility to apply this strategy to control soil borne epidemics. For instance, synthetic pure isothiocyanates resulted active *in vitro* in assays against *Sclerotinia sclerotiorum*, especially in reducing radial growth of mycelium, scleriotia viability and germination (Kurt *et al.*, 2011). Under field conditions, the activity of biofumigation is variable due to plant tissues, temperatures and soil moistures (Gimsing and Kirkegaard, 2009). The use of some *Brassica* species as cover crops reduced the number of *Verticillium dahliae* microsclerotia as well as the disease severity in the following crops (Xiao *et al.*, 1998), however,

the results obtained were not consistent in other experiments (Hartz *et al.*, 2005). Beneficial effects on yield and protection against *S. minor* disease were observed in lettuce crops grown after mustard and other cover crops (Bensen *et al.*, 2009; Hao *et al.*, 2003).

3.3.4 Organic amendments and compost

Organic amendments such as animal manures and compost are commonly used in agricultural production to increase soil fertility. Their utilization also provides additional benefits such as improved plant heath due to the reduction of pathogen inoculum (Bailey and Lazarovits, 2003; Gamliel *et al.*, 2000; Lazarovits, 2001; Lazarovits *et al.*, 2001) . The liberation of volatile ammonia following application of amendments is responsible for killing pathogens (Bailey and Lazarovits, 2003). For instance, the production of ammonia and nitrous acid as consequence of degradation of these amendments resulted toxic to *Verticillium dahliae* microsclerotia (Tenuta and Lazarovits, 2002).

Compost is obtained by biological decomposition of organic materials, which determines their chemical stabilization and the sanitization from human and plant pathogens and weed seeds (Noble and Roberts, 2004). During the cooling phase of the process, microbial community naturally recolonizes the compost and acts as antagonist against pathogens (Hadar and Papadopoulou, 2012). Compost from urban organic or green wastes is reported to control Fusarium wilt, *Pythium* spp. and other soil borne pathogens (Alfano *et al.*, 2011; Pascual *et al.*, 2002; Ros *et al.*, 2005).

The effectiveness of organic amendment as well as compost is variable: changing methods of residue management influences the microbial community as well as other unknown factors in soil (Bailey and Lazarovits, 2003; Chellemi, 2002; Gamliel *et al.*, 2000)

4 Biological control of soil borne pathogens

4.1 Definition

The terms “biological control” and its abbreviated synonym “biocontrol” have been used in different fields of biology, most notably in entomology and plant pathology. In plant pathology, biological control is defined as “the reduction in the amount of the inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man” (Cook and Baker, 1983). The organism that suppresses the pathogen is called Biological Control Agent (BCA).

In certain areas, the antagonistic microorganisms are part of the indigenous micro-flora and these soils are called “suppressive soils”. Otherwise, the antagonistic microorganisms can be introduced in soil through seed treatments, soil applications, roots dip and drip irrigation (Warrior *et al.*, 2002).

4.2 Suppressive soils

In certain areas, although the pathogen is present and the plant host is susceptible, the disease incidence or severity appears lower than expected. These areas are defined suppressive soils (Mazzola, 2002). Many suppressive soils have been recognized and characterized for numerous plant-pathogen systems, but the most studied are those caused by *Fusarium* spp. (Alabouvette, 1986) and *Gaeumannomyces graminis* var. *tritici* (Simon and Sivasithamparam, 1989), the causal agents of Fusarium wilt and take-all of wheat, respectively. The factors that cause the suppressiveness are both abiotic and biotic. Chemical and physical attributes such as pH and clay content influence the disease suppression (Amir and Alabouvette, 1993). However, the indigenous microbial community plays a significant role in disease suppression (Weller *et al.*, 2002). Indeed, the suppressiveness is lost if the soil is pasteurized. In Fusarium wilt suppressive soil, non-pathogenic *Fusarium* spp. and fluorescent pseudomonads act in concert to suppress the disease (Weller *et al.*, 2002). The mechanisms involved are the production of siderophores, the saprophytic competition for substrate and the induction of systemic resistance in the plant host (Couteaudier and Alabouvette, 1990; Raaijmakers *et al.*, 1995).

In some monoculture systems, the spontaneous disease decline has been observed. For instance, in wheat cropping, the take-all decline is attributed to increased population of certain fluorescent *Pseudomonas* which produce 2,4 diacetylphloroglucinol (Raaijmakers *et al.*, 1999). Similarly, the decline in the severity of disease caused by *Rhizoctonia solani* has been documented in response to successive planting of given plant host. This effect has been attributed to increased parasitism by *Trichoderma harzianum* (Liu and Baker, 1980).

4.3 Modes of action of BCAs

Biological control can result from many different types of interaction between the organisms. In all cases, the pathogen is antagonized by the presence and activities of other organisms.

In hyperparasitism, the pathogen is directly attacked by a specific BCA that kills it or its propagules. For instance, the fungus *Coniothyrium minitans* parasitizes and kills *Sclerotinia* spp. sclerotia (Campbell, 1947; Jones *et al.*, 2004), *Trichoderma* spp. can parasitize a range of other fungi thanks to the production of several fungal cell-wall degrading enzymes (Harman *et al.*, 2004) and the virus that infects *Cryphonectria parasitica* causes hypovirulence of the pathogen (Milgroom and Cortesi, 2004). Antibiosis is the antagonism resulting from the production by one microorganism of secondary metabolites toxic to other microorganisms. It is a common phenomenon responsible for the activity of many BCAs such as fluorescent *Pseudomonas* spp., *Bacillus* spp., *Streptomyces* spp., and *Trichoderma* spp. (Haas and Defago, 2005; Raaijmakers *et al.*, 2002). The metabolites include not only antibiotics *sensu stricto*, but also cell wall degrading enzymes, and volatile compounds with antifungal activity. Many microorganisms produce and release lytic enzymes that can hydrolyze a wide variety of polymeric compounds, including proteins, cellulose and hemicellulose. The biocontrol agent *Streptomyces lydicus* WYEC108 produces cell wall degrading enzymes (Mahadevan and Crawford, 1997). Competition for nutrients is another mechanism. The competition occurs between pathogenic and non-pathogenic *Fusarium oxysporum* for the carbon source (Couteaudier and Alabouvette, 1990). Competition for minor elements also occurs: iron is an essential micronutrient but is extremely limited in the rhizosphere, depending on soil pH. Some microorganisms can secrete iron-binding ligands called siderophores having high affinity to sequester iron from the mico-environment. (Boukhalfa and Crumbliss, 2002). Direct correlation was established *in vitro* between siderophores synthesis in fluorescent pseudomonads and their capacity to inhibit germination of chlamydospores of *F. oxysporum* (Loper and Buyer, 1991).

4.4 Limits of biological control

In the last years, numerous studies were done to identify BCAs and to investigate their mechanisms of action. Some BCAs are now available on the market and applied in horticultural farms. For instance, the fungus *Coniothyrium minitans* is the antagonist microorganism present in the product Contans (Intrachem, Italy) used to control *Sclerotinia* diseases as well as *Bacillus amyloliquefaciens* D747 (Amylo-X, Intrachem, Italy). *Streptomyces griseoviridis* K61 (Mycostop, Verdera, Finland) is used to control root rots on vegetable crops and also some products based on *Trichoderma* spp. are present. However, the number and application range of BCAs is quite limited, because once applied in field they often lose their efficacy in controlling the target pathogen. Several reasons can explain

this lack of consistency. They show a narrow specificity, therefore their application will not affect the non-target pathogen genotypes. Since the population of the pathogenic organism presents a certain diversity and a single given strain of a BCA might not have the same efficacy on all pathotypes present in the population (Schisler *et al.*, 2000), biocontrol efficacy varied upon genotype diversity of the target population. The antagonists are often extensively studied in laboratory in simplified environments such as growth chambers, experimental greenhouses or small field plots, thus avoiding the risk of large-scale experiments (Spadaro and Gullino, 2005), but the eco-climatic conditions in nature are much more variable than those in the laboratory and this could result in a lower control of the pathogen (Alabouvette *et al.*, 2006).

The biology and the modes of action of BCAs are also important to better understand how to apply the antagonist. For instance, the stains with a mechanism of action of antibiosis have to be applied at the right place and the right time, because the secondary metabolites are not produced in great quantities and might not be transported at great distances (Alabouvette *et al.*, 2006).

5 Streptomycetes

5.1 Taxonomy

The genus *Streptomyces* was introduced by Waksman and Henrici in 1943. They are Gram-positive aerobic bacteria, members of the order *Actinomycetales* within the class *Actinobacteria* and have a DNA guanine and cytosine content of 69-78 mol% (Korn-Wendisch *et al.*, 1992).

The taxonomy of the genus *Streptomyces* has been of interest for researchers from 1940s when the discovery of antibiotics produced by streptomycetes led to extensive screening for novel compounds. Consequently, the need for patenting led to an overclassification of the genus (Anderson and Wellington, 2001). Species described within the genus *Streptomyces* increased from approximately 40 to over 3000, but many of these strains were considered to be synonyms. To prevent overspeciation, standard identification criteria and type strains were needed. For these reasons, in 1964, the International *Streptomyces* Project (ISP) was initiated to introduce standard criteria for the determination of species so as to reduce the number of poorly described synonymous species. The criteria were based on morphological features such as mycelia, soluble pigments, spore chain, spore surface, production of melanin pigment, and the utilization of a range of carbon sources (Shirling and Gottlieb, 1968a; b; 1969; 1972; Tresner and Backus, 1963). The strain characterization was carried out using specific culture medium prepared for ISP by Difco Laboratories (USA). More than 450 *Streptomyces* species were re-described and type strains were selected and deposited in internationally recognized culture collections. In 1980s and in following years, the numerical taxonomy was introduced (Kampfer *et al.*, 1991; Langham *et al.*, 1989; Williams *et al.*, 1983). According to this method, streptomycetes were analyzed for the phenotypic traits and the utilization of carbon source and subsequently clustered on the basis of similarities and differences using specific algorithms. Each cluster is regarded as single species or species-group. Using the numerical classification approach, the 1989 edition of Bergey's Manual describes 142 species (Locci, 1989), in contrast to 463 species described in the 1974 edition (Pridham and Tresner, 1974).

The application of molecular techniques to the analysis of bacterial genomes has contributed considerably to the knowledge of *Streptomyces* genus taxonomy and many methods can be applied to study the strepomycete strains (Anderson and Wellington, 2001).

Despite the numerous studies, nowadays the taxonomy of *Streptomyces* genus remains somewhat confused because of the lack of correlation between the phenotypic and genotypic approach for streptomycetes identification. This emphasizes the importance of a polyphasic taxonomic approach, which can only be maintained by standardization and collaboration so that molecular, biochemical

and phenotypic traits can be weighted and examined in an integrative manner (Anderson and Wellington, 2001).

5.2 Life cycle

Streptomycetes have a life cycle unique among bacteria. It consists of two growth steps: the vegetative and the sporogenous phases. When these organisms are grown on an adequate solid medium, the spores germinate and grow. The germination can be divided in three sequential stages: darkening, swelling and germ tube emergence. During these steps both morphological and biochemical events occur (Hardisson *et al.*, 1985b; Hardisson *et al.*, 1978; Salas *et al.*, 1985). Later, the germ tube grows and forms a mat of hyphae firmly attached to the solid surface. This represents the substrate or vegetative mycelium. Subsequently, when the nutritional conditions start to be adverse for the vegetative growth, specialized aerial hyphae arise on the top of the mycelium, originating the aerial mycelium, which uses the nutrients for its development from the vegetative one. This mycelium forms chains of three to many spores called sporophores at maturity (Locci, 1989). They are formed by formation of cross-walls in the multinucleate aerial filaments followed by separation of individual cells (Wildermuth and Hopwood, 1970).

5.3 Morphology

When grown in medium, streptomycete colonies form a discrete and lichenoid, leathery or butyrous colonies. Initially, the colonies are relatively smooth surfaced, but later they develop a weft of aerial mycelium that may appear floccose, granular, powdery, or velvety (Locci, 1989) (Figure 5.3.1 and Figure 5.3.2). Streptomycetes produce a wide variety of pigments responsible for the color of the vegetative and aerial mycelia. Colored diffusible pigments may also be formed.

The life cycle of streptomycetes offers three features for morphological characterization: a) the vegetative mycelium, b) the aerial mycelium bearing the sporophores, and c) the spores themselves.

The vegetative mycelium is constituted by thin hyphae (0.5-2 μm in diameter) that often lack cross-walls and are extensively branched. Depending on the temperature, the pH and the age of culture the substrate mycelium can show numerous colors and soluble pigments: blue, dark green, red, and violet (Baldacci *et al.*, 1954). Regarding the aerial mycelium, the range of spore chain (sporophores) morphology is extensive. Three categories are recognized: straight to flexuous (*Rectiflexibles*), hooks, loops, or spirals with one to two turns (*Retinaculiaperti*), and spirals (*Spirales*) (Shirling and Gottlieb, 1966). Moreover, numerous spore mass colors were recognized: blue, gray, green, red, violet, white, and yellow (Shirling and Gottlieb, 1966). Lastly, the spores show different morphologies, especially in their surface. Under scanning electron microscopy,

different categories can be recognized: smooth, spiny, hairy, warty, and rugose (Figure 5.3.2) (Dietz and Mathews, 1971; Tresner *et al.*, 1961).

Figure 5.3.1 – *Streptomyces* colony observed at scanning electron microscope.

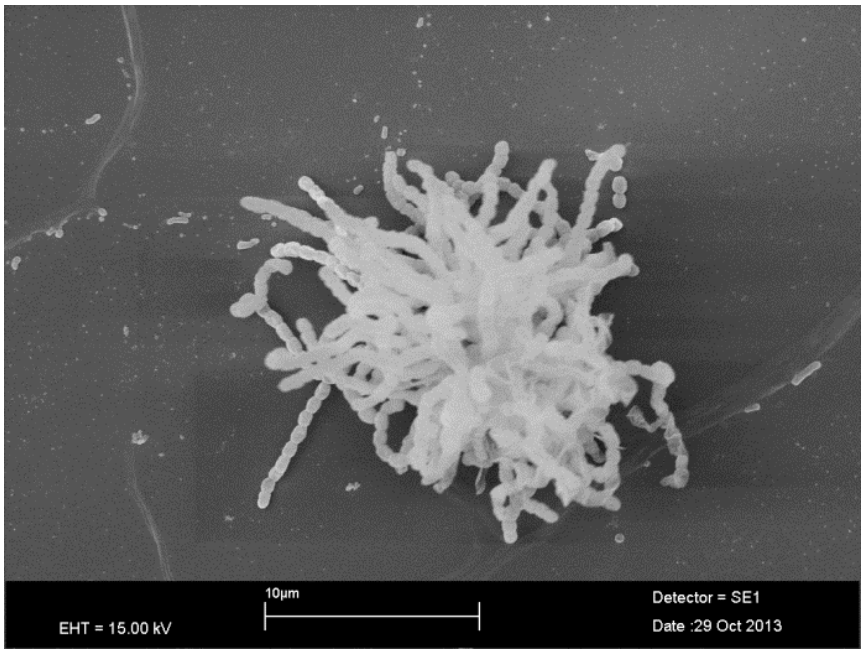
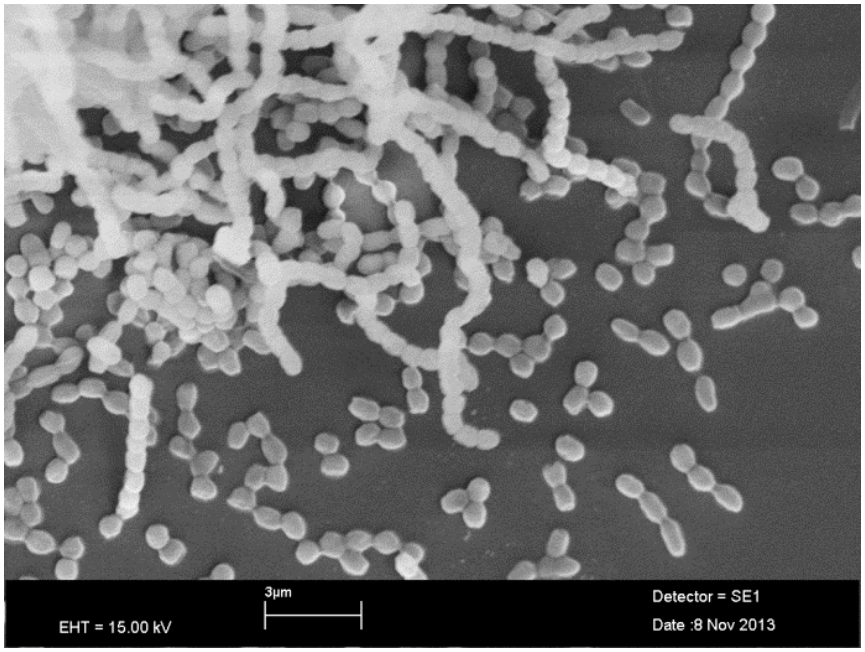


Figure 5.3.2 – *Streptomyces* sporophores and spores observed at scanning electron microscope.



5.4 Antibiotics production

Concomitant with the morphological switch from vegetative to aerial mycelium, the production of antibiotics begins. This fact could support the role of antibiotics to protect the vegetative mycelium

from other microorganisms present in the soil, thus preserving them as a source of nutrients for aerial growth (Chater and Bibb, 1997; Hardisson *et al.*, 1985a). Streptomycetes are the largest antibiotics-producing genus in the microbial world (Watve *et al.*, 2001). Streptothricin, actinomycin and streptomycin were the first discovered antibiotics produced by *Streptomyces* spp. (Schatz *et al.*, 1944; Waksman, 1943; Waksman and Tishler, 1942). From 1950s to 1970s started the screening of streptomycetes for antibiotics production and a wide number of compounds were found and characterized, for instance: novobiocin, vancomycin, tetracycline, nystatin (Behal, 2000; Hopwood, 2007; Watve *et al.*, 2001). They have antibacterial, antifungal and anticancer activities and are applied in pharmaceutical products for human health (Doumbou *et al.*, 2001; Hopwood, 2007).

5.5 Streptomycetes in soil and their relationship with plant roots

Streptomycetes are ubiquitous in nature and are commonly found in soil where they are responsible for the typical "earthy odor" due to the production of geosmin (Gerber and Lechevalier, 1965). Moreover, they are involved in the decomposition of lignin and cellulose (Crawford, 1978) and in the break-down of organic matter in soil (Goodfellow and Simpson, 1987).

Few species are known as plant pathogens: *Streptomyces scabies* is the most important worldwide. It is the agent of common scab of potato, causes superficial and erumpent lesions, most of which have a raised, rough, corky appearance (Lambert and Loria, 1989). Other minor pathogens are known in some areas of the world, such as *S. acidiscabies*, which produces symptoms like those of *S. scabies* on potato and on taproot crops and *S. ipomoeae* that causes soil rot of sweet potato (Loria *et al.*, 1997). The phytotoxin thaxtomin was discovered to play a critical role in the pathogenicity of streptomycetes. It inhibits cellulose biosynthesis in plant cells and causes cell death (Fry and Loria, 2002; Loria *et al.*, 2006).

Streptomycetes are found to colonize rhizosphere, enter the root tissues and establish endophytic lifestyle with plants (Cao *et al.*, 2004; Coombs and Franco, 2003a; Petrolini *et al.*, 1996; Sardi *et al.*, 1992). Due to production of a wide number of antifungal compounds (Doumbou *et al.*, 2001) and chitinase (Mahadevan and Crawford, 1997; Taechowisan *et al.*, 2003a), the ability of some streptomycete strains to inhibit plant pathogens and therefore act as promising biological control agents has been investigated. However, though streptomycete strains have been extensively characterized for secondary metabolite production, studies on their relationship with plants are poorly studied. Some strains were screened and characterized for their activity against soil-borne pathogens. For instance, they were studied against *Pythium* seed and root rot (Yuan and Crawford, 1995), *Phytophthora* root rot (Xiao *et al.*, 2002), *Rhizoctonia* damping-off of tomato (Sabaratnam and Traquair, 2002) and *Sclerotinia* basal drop (Bonaldi *et al.*, 2014; El-Tarabily *et al.*, 2000). Two strains were developed in commercial products and are available on the market: Mycostop® is

based on *S. griseoviridis* strain K61 (Verdera, Finland) and Actinovate®SP based on *S. lydicus* strain WYEC 108 (Natural Industries Inc., Houston, TX).

Streptomycetes have been little investigated as Plant Growth Promoting Bacteria (PGPB). Some works were carried during the 1980th - '90th at the University of Milan (Quaroni *et al.*, 1997; Saracchi *et al.*, 1991) and only recently, the interest on streptomycete beneficial effects on plant growth is gaining increased attention; their positive effects on root nodulation in Pea plants were observed (Tokala *et al.*, 2002b), as well as the increase of fresh and dry weight and length of roots and shoots of bean (Nassar *et al.*, 2003).

Outline and aim of the work

Leafy vegetables are economically important crops in the Italian horticulture. Due to the intensive cultivation systems, salad crops are particularly exposed to the risk of phytopathological problems especially those caused by soil borne pathogens. They represent a serious danger for the grown of leafy vegetable from their initial stages of cultivation up to harvest, and often caused important economic and yield losses. For many years the management of soil borne epidemics mainly relied on application of soil fumigants. However, the new European regulations have seriously restricted the use of chemicals in crop protection encouraging other non-chemical approaches. Among the integrated pest/disease management strategies, the application of Biological Control Agents (BCAs) represents a valuable component.

The use of BCAs to manage soil borne epidemics is gaining more and more interest in modern agriculture as confirmed by the numerous researches to develop bacterial and fungal strains as biopesticides. Nowadays some of them are commercial products and are applied in field to reduce disease incidence or severity. However, they often show variable performance and the crops protection is not always as effective as it should be expected.

Identifying new antagonist microorganisms is of fundamental importance in horticulture and increasing the knowledge about their biology, such as mechanisms of action, and their modes of application is essential to obtain the best performance.

Bacteria of the genus *Streptomyces* represent a significant fraction of the soil microflora. They can establish beneficial relationships with plants by colonizing the rhizosphere and entering the root tissues. These features together with the wide number of antifungal compounds they produce, make streptomycetes promising antagonists of soil borne pathogens.

This research project aims to study *Streptomyces* spp. as BCAs and as Plant Growth-Promoting Bacteria for the protection and cultivation of salad crops.

Two hundred endophytic *Streptomyces* strains were involved in the work (Chapter 1) and the project was organized in different steps:

- Chapter 2: a massive selection of strains was carried out by dual culture assays. The bacteria were analyzed for their ability to inhibit the mycelium growth of six fungi, main agents of soil borne diseases.
- Chapter 3: *Streptomyces* strains were characterized for the principal traits involved in the Plant Growth Promotion.

- Chapter 4: a pool of *Streptomyces* strains which showed strong antagonism *in vitro* against *Sclerotinia sclerotiorum* was studied in growth chamber and field for their efficacy to reduce lettuce drop incidence, primarily searching for the potential influence of the antagonist application timing.
- Chapter 5: the interaction antagonist-plant host was studied as the spatio-temporal colonization dynamics of lettuce roots and rhizosphere with an EGFP-labelled *Streptomyces* strain.

1 Chapter 1: *Streptomyces* strains

1.1 Introduction

A wide collection of 1755 endophytic actinomycetes is stored in the laboratory of Plant Pathology at the Department of Food, Environment and Nutritional Sciences (DeFENS), University of Milan. Among them, 1422 isolates were identified as *Streptomyces* spp. (Petrolini *et al.*, 1996). Strains were isolated by sampling roots of 156 plant species showing healthy vegetative growth, during a 7 year period (1987-1994). Altogether 205 plants, 50 of which grapevines *(Vitis vinifera),* were collected from different habitats and locations in northern Italy. Roots (1 to 5 mm in diameter) were washed to remove soil particles, and were surface sterilized by exposing them to propylene oxide vapors for 1 h. Then, aseptically cut pieces (about 10 mm) were incubated for up to 21 days at 25°C on WA medium (Sardi *et al.*, 1992). For each root sample, the largest number of streptomycete colonies showing different morphological characteristics was isolated. Cultures were preserved by lyophilization and/or by freezing spore suspensions in 10% (w/v) glycerol at -20°C. CZY medium was chosen as a suitable medium favoring sporu1ation (Petrolini *et al.*, 1996). Strains were labelled according to plant species isolation sources.

1.2 *Streptomyces* culture

In this PhD work, 200 strains, out of the 1755 of the collection, were randomly chosen for the goal of the work (Table 1.3.1). From the original stock, strains were inoculated on CZY medium and incubated for 3 weeks at 24°C until their sporulation. Spores of each strain were collected in 10% sterile glycerol and spore suspension (10^7 spores/mL) was stored at -20°C.

1.3 Reference strain

In the *in vitro* and in field experiments, the activity of streptomycetes was compared to Actinovate (Natural Industries, Inc. Houston) a product based on *Streptomyces lydicus* WYEC 108. From the commercial product, the strain was isolated through serial dilutions on CZY medium added with 50 mg/L nystatin and 50 mg/L cycloheximide to suppress fungal growth. After ten days growth on Petri plates, spores were collected in 10% sterile glycerol and spore suspension (10^7 spores/mL) was stored at -20°C.

Table 1.3.1– Streptomycete strains and relative plant species isolation source.

Strain	**Crop species**	**Strain**	**Crop species**
ALC03R	*Allium cepa*	CN08W	*Phragmites communis*
ALG06R	*Alnus glutinosa*	CN09W	*Phragmites communis*
ALG07R	*Alnus glutinosa*	CN13W	*Phragmites communis*
ALP07R	*Allium porrum*	CPB02R	*Capsella bursa pastoris*
ALP10R	*Allium porrum*	CPB08R	*Capsella bursa pastoris*
ALP11R	*Allium porrum*	CPB11R	*Capsella bursa pastoris*
AM10A	*Amaryllis belladonna*	CR13A	*Chrysanthemum indicum*
AM12A	*Amaryllis belladonna*	CRC04R	*Crocus* sp.
AM27W	*Amaryllis belladonna*	CRM05R	*Cornus mas*
ARF07R	*Arthrocnemum fruticosum*	CRM14R	*Cornus mas*
ARF09R	*Arthrocnemum fruticosum*	CRM31R	*Cornus mas*
ARF16R	*Arthrocnemum fruticosum*	CSM12R	*Cornus mas*
ARF24R	*Arthrocnemum fruticosum*	CU07W	*Cyclamen persicum*
ARN01R	*Arbutus unedo*	CVM02R	*Brassica oleracea*
ARN02R	*Arbutus unedo*	CX08W	*Carex* sp.
ARN08R	*Arbutus unedo*	CX14W	*Carex* sp.
ARN09R	*Arbutus unedo*	CX16W	*Carex* sp.
AST32I	*Aster* sp.	CX17W	*Carex* sp.
AZ112I	*Azalea* sp.	EM05W	*Medicago sativa*
AZ117I	*Azalea* sp.	EP03W	*Euphorbia* sp.
AZ144I	*Azalea* sp.	EP05W	*Euphorbia* sp.
BT08A	*Betula pendula*	EP07W	*Euphorbia* sp.
BT15W	*Betula pendula*	EP11W	*Euphorbia* sp.
BT17W	*Betula pendula*	EP17A	*Euphorbia* sp.
BT28W	*Betula pendula*	EPH11R	*Euphorbia* sp.
BT29W	*Betula pendula*	EPH21R	*Euphorbia* sp.
CHL01R	*Chelidonium majus*	EPH36R	*Euphorbia* sp.
CM16A	*Camellia japonica*	EQS04R	*Equisetum arvense*
CM20A	*Camellia japonica*	ER18A	*Erica carnea*
CMJ57I	*Camellia japonica*	ER19A	*Erica carnea*
CMJ58I	*Camellia japonica*	ER20A	*Erica carnea*
CMJ60I	*Camellia japonica*	EW13W	*Medicago sativa*
CN05W	*Phragmites communis*	EW15W	*Medicago sativa*
CN06W	*Phragmites communis*	EW16W	*Medicago sativa*

Strain	Crop species	Strain	Crop species
FA01W	*Fragaria vesca*	LT05W	*Lactuca scariola* var. *sativa*
FA05W	*Fragaria vesca*	LYC01E	*Lycopersicum esculentum*
FA07W	*Fragaria vesca*	LYC02E	*Lycopersicum esculentum*
FIC11R	*Ficus carica*	MR01W	*Vaccinium myrtillus*
FIC35R	*Ficus carica*	MR02W	*Vaccinium myrtillus*
FR05W	*Fragaria* x *ananassa*	MR05W	*Vaccinium myrtillus*
FS02W	*Festuca rubra*	MR11W	*Vaccinium myrtillus*
FS12W	*Festuca rubra*	MR13W	*Vaccinium myrtillus*
FS18W	*Festuca rubra*	MR16W	*Vaccinium myrtillus*
FS26W	*Festuca rubra*	MR19W	*Vaccinium myrtillus*
FT04W	*Triticum aestivum*	MR20W	*Vaccinium myrtillus*
FT05W	*Triticum aestivum*	MR24W	*Vaccinium myrtillus*
FT06W	*Triticum aestivum*	MRX13R	*Rumex* sp.
GC02W	*Hyacinthus orientalis*	MRX44	*Rumex* sp.
GC03W	*Hyacinthus orientalis*	NPH11R	*Nuphar* sp.
GNL02R	*Gentiana lutea*	OCB07R	*Ocymium basilicum*
HLC02R	*Heleocharis* sp.	OCB10R	*Ocymium basilicum*
HLP01R	*Halimione portulacoides*	OCB15R	*Ocymium basilicum*
HLP03R	*Halimione portulacoides*	OCB21R	*Ocymium basilicum*
HLP08R	*Halimione portulacoides*	PLM01R	*Polygonatum moltiflorum*
HYP03R	*Hypericum* sp.	PLR02R	*Pelargonium* sp.
HYP23R	*Hypericum* sp.	PO03W	*Allium porrum*
KAL01R	*Kalanchoe* sp.	PO07W	*Allium porrum*
LAU18R	*Laurus* sp.	PRT06R	*Parietaria officinalis*
LM01W	*Rubus idaeus*	PRT07R	*Parietaria officinalis*
LM07W	*Rubus idaeus*	PRV04R	*Prunus avium*
LM08W	*Rubus idaeus*	PTH08R	*Petroselinum hortense*
LMN06R	*Limonium* sp.	QR03W	*Quercus* sp.
LMP64I	*Rubus idaeus*	QR06W	*Quercus* sp.
LMP72I	*Rubus idaeus*	QR16W	*Quercus* sp.
LMP74I	*Rubus idaeus*	QR19W	*Quercus* sp.
LRS17R	*Laurus nobilis*	QR26W	*Quercus* sp.
LRS20R	*Laurus nobilis*	QR29W	*Quercus* sp.
LRS39R	*Laurus nobilis*	RBF05R	*Rubus fruticosus*
LRS40R	*Laurus nobilis*	RBF10R	*Rubus fruticosus*
LRS45R	*Laurus nobilis*	RFB14A	*Rubus fruticosus*

Strain	Crop species	Strain	Crop species
RMX14R	*Rumex* sp.	SYS13R	*Sisymbrium officinale*
RMX17R	*Rumex* sp.	TAG17R	*Tagetes* sp.
ROS77F	*Rosa* sp. “Blue Moon”	TOR01L	*Erica carnea*
RSM08R	*Rosmarinus officinalis*	TOR16L	*Erica carnea*
RSM10R	*Rosmarinus officinalis*	TOR51L	*Erica carnea*
SG04W	*Secale cereale*	TOR57I	*Erica carnea*
SG06W	*Secale cereale*	TOR62L	*Erica carnea*
SG09W	*Secale cereale*	TOR65I	*Erica carnea*
SG10W	*Secale cereale*	TRX03R	*Taraxacum officinale*
SG12W	*Secale cereale*	TXB01R	*Taraxacum officinale*
SJ01W	*Glicine max*	TXB16R	*Taraxacum officinale*
SJS02R	*Glicine max*	TXB24R	*Taraxacum officinale*
SLF27R	*Salvia officinalis*	VLA11R	*Viola tricolor*
SLP02R	*Silene apetala*	VLA34R	*Viola tricolor*
SN02A	*Saintpaulia kewensis*	VNC12R	*Vinca major*
SPJ01R	*Spartium junceum*	VRN01R	*Veronica* sp.
ST06W	*Glicine max*	VT041R	*Vitis vinifera*
ST07W	*Glicine max*	VT098I	*Vitis vinifera*
SUA02R	*Suaeda* sp.	VT101I	*Vitis vinifera*
SUA03R	*Suaeda* sp.	VT104I	*Vitis vinifera*
SW01W	*Glicine max*	VT105I	*Vitis vinifera*
SW06W	*Glicine max*	VT111I	*Vitis vinifera*
SW27W	*Glicine max*	VT334R	*Vitis vinifera*
SW29W	*Glicine max*	VT394R	*Vitis vinifera*
SW35W	*Glicine max*	VTV06R	*Vitis vinifera*
SW37W	*Glicine max*	YRU27D	*Vitis vinifera*
SW42W	*Glicine max*	YSO07R	*Sonchus* sp.
SW46W	*Glicine max*	ZEA07I	*Zea mays*
SYS01R	*Sisymbrium officinale*	ZEA13I	*Zea mays*
SYS08R	*Sisymbrium officinale*	ZEA17I	*Zea mays*

2 Chapter 2: Dual culture assay for selection of *Streptomyces* strains as potential Biological Control Agents (BCAs) of soil borne pathogens.

2.1 Introduction

Among the modes of action of bacterial Biological Control Agents (BCAs) of fungal pathogens, antibiosis is one of the most widely used (Pal and McSpadden Gardener, 2006). It refers to the production of secondary metabolites that interfere with the target pathogen growth resulting in a reduction of disease incidence or severity. The compounds include molecules with antifungal properties, lytic enzymes that degrade the fungal cell wall and siderophores that bind iron, thus limiting pathogen growth (Loper and Buyer, 1991; Nagarajkumar *et al.*, 2004; Raaijmakers *et al.*, 2002). When bacterial isolates are searched as BCAs, dual culture assays often represent the first step of the work (Pliego *et al.*, 2011). The tests are typically carried out in agar plate and this approach permits massive screening of numerous strains. According to this method, the candidate bacteria and the pathogen are co-cultured on the same agar plate. The antifungal compounds released by the active bacterial strain inhibit the mycelium growth and the antagonistic activity of the bacterium is expressed as the reduction of fungal colony growth. The potential bacterial BCAs are typically ranked according to this ability.

Subsequently, for the most active strains, further assays can be performed to better investigate if lytic enzymes or other compounds are involved in the antagonistic activity. The main lytic enzymes produced by bacterial BCAs are chitinases, glucanases and proteases. Specific growing media can be prepared to study the enzymes production. For instance, medium containing colloidal chitin as single carbon source is used to study the production of chitinases (Taechowisan *et al.*, 2003a). If bacterial isolate releases the enzyme, a clear halo is formed below its colony. Specific medium can be used to detect the siderophore production (Schwyn and Neilands, 1987). Briefly, bacterial cultures are grown on media devoid of iron. Addition of specific reagents causes color change of the growing medium around the siderophore producing-colony (Pérez-Miranda *et al.*, 2007).

Many bacterial strains belonging to different genera have been screened with dual culture assay and their activity against several pathogens has been analyzed (Ashwini and Srividya, 2014; Bano and Musarrat, 2003; Kalbe *et al.*, 1996). Also strains of the genus *Streptomyces* have been studied using this approach (Boukaew *et al.*, 2011; de Vasconcellos and Cardoso, 2009; Taechowisan *et al.*, 2003b). However, in spite of numerous applications of dual culture tests, the methodologies such as timing and mode of microorganism inoculation have never been considered as a crucial point and therefore investigated. Moreover, the methods are not standardized and can vary among the studies reported in literature. Bacterial strains are often inoculated 3-8 days before the pathogen to favor the

production of secondary metabolites (Chamberlain and Crawford, 1999; Crawford *et al.*, 1993; Khamna *et al.*, 2009). Also the distance between the pathogen and the antagonist on the agar plate can vary from 2.5 to 5 cm (Boukaew *et al.*, 2011; Trejo-Estrada *et al.*, 1998). Finally, it is well known that fungal strains differ in growth in agar medium. To be able to compare the activity of a single antagonist against more than one fungal strain, it is fundamental to standardize the distance antagonist-pathogen for each fungus for a better evaluation of the inhibition activity.

The aim of this work was to analyze a collection of streptomycete isolates as antagonist microorganisms of six soil borne fungal pathogens: first, the dual culture assay was optimized for each pathogen, and, second, optimized dual culture assay for each antagonist-pathogen combination was used to screen the streptomycete collection.

2.2 Methodology

2.2.1 *Streptomyces* culture

Two-hundred *Streptomyces* strains (see Paragraph 1.2 for details) were tested for their antibiosis activity against six fungal pathogens. *Streptomyces lydicus* WYEC 108, the strain contained in the commercial product Actinovate, was used as reference strain (see Paragraph 1.3 for details). Streptomycetes were inoculated as agar-spore suspension. Briefly, 10 µL of spore suspension (10^7CFU/mL) were added to 90 µL of 0.2% sterile water agar and 10 µL of agar-spore suspension was streaked (40 mm) on the agar plate.

2.2.2 Fungal culture

The fungi used in this work represent the main soil borne pathogens of horticultural crops. Four of them are part of a collection of fungi stored in the laboratory of Plant Pathology at the Department of Food, Environment and Nutritional Sciences (DeFENS), University of Milan. The isolates are: *Sclerotinia sclerotiorum, Fusarium oxysporum* f.sp. *lactucae, Thielaviopsis basicola* and *Pythium ultimum*. The additional two species, *Rhizoctonia solani* and *Phytophthora* sp., were kindly provided by Dr. Andrea Minuto (Centro di Sperimentazione e Assistenza Agricola, Albenga, Italy). The fungi were maintained at 20°C on MEA medium whereas. *Phytophtora* sp. on V8.

2.2.3 Dual culture assay: medium and growing conditions

The assay was performed in Petri plates (90 mm diameter). CZY medium was used for all fungi, only for *T. basicola*. the antibiosis test was performed on PDA medium. Following pathogen-antagonist inoculation, the plates were incubated at 24°C in the dark.

2.2.4 Assessment of antagonist inhibition activity

Antagonism of each streptomycete strain was determined by calculating the percentage of the growth inhibition of the fungus mycelium. For each strain three replicates were prepared. Three plates inoculated with the pathogen only were used as control. The percentage of inhibition was determined by the Equation 1: (R1-R2)/R1 x 100, where R1 was the average of the mycelium radial growth on control plates and R2 was the average of the mycelium radial growth of the pathogen in the presence of the antagonist.

2.2.5 Dual-culture assay optimization

Fungal growth curves

A mycelium-disc (6 mm diameter), taken from the edge of an actively growing fungal colony, was inoculated in the middle of a Petri plate containing CZY or PDA medium. Plates were incubated at 24°C in the dark. Four perpendicularly radial mycelium measures were taken from the edge of the disc. The measures were done daily for 7 days in three replicates. Daily, the growth was calculated averaging the distances. Subsequently, according to the growth curves, pathogens were grouped as fast, medium and slowly growing, group 1, 2 and 3, respectively.

Antagonist inoculation timing

To study the effects on inhibition rate due to different antagonist inoculation times, the timing of pathogen-antagonist inoculation was studied using CMJ57I and CX14W as steptomycete reference strains and *S. sclerotiorum* and *F. oxysporum* f.sp. *lactucae* as reference fungi. A mycelium-agar disc (6 mm diameter) was placed in the middle of a Petri plate containing CZY. Streptomycete strain was inoculated at a standardized distance from the edge of the mycelium-agar disc according to the mycelium growth curves (Table 2.3.1 and Table 2.2.1). Based on pathogen inoculation timing, four trials were prepared:

A. *Streptomyces* strain and pathogen inoculated the same day
B. *Streptomyces* strain inoculated 1 day before the pathogen
C. *Streptomyces* strain inoculated 2 days before the pathogen
D. *Streptomyces* strain inoculated 3 days before the pathogen

For each combination three replicates were prepared. Three plates inoculated with the pathogen only were used as control. Daily mycelium radial growth was measured perpendicularly to the streptomycete colony and in the control plates. The daily percentage of inhibition was calculated for each streptomycete strain. The percent data were arcsine root-squared transformed. The data were submitted to ANOVA, followed by a Tukey post-hoc test for multiple comparison (P =0.05), using the TukeyC packages (Faria *et al.*, 2013) using R software, version R3.0.2 (R_Core_Team, 2013).

Interval of inhibition assessment

To determine the optimal interval of growth to estimate the best mycelium growth inhibition, dual culture were performed for a pathogen belonging to each group of growth and CMJ57I and CX14W were used as streptomycete reference strains. In plates containing CZY or PDA, the antagonist and the fungus were inoculated at different times and distances (Table 2.2.1). Every day the mycelium radial growth was measured perpendicularly to the streptomycete colony and in control plates. The percentage of inhibition was calculated as described previously.

Table 2.2.1 –Parameters of the dual culture assay for each group of fungi.

Group	**Distance streptomycete-fungus (mm)**	**Time of pathogen inoculation after streptomycete**	**Days of inhibition measurement after pathogen inoculation**
Group 1	25	Two days after	3
Group 2	20	The same day	6
Group 3	10	The same day	7

2.2.6 Screening of Streptomycete strains

Two hundred streptomycete strains were analyzed for their antagonistic activity against the six soil borne pathogens applying the standardized dual culture assay (see Paragraph 1.2 for details). The inhibition activity of tested strains was compared to *Streptomyces lydicus* WYEC 108 (see Paragraph 1.3 for details).

2.3 Results

2.3.1 Dual-culture assay optimization

Fungal growth curves

The fungi *S. sclerotiorum* and *R. solani* showed the fastest growth: four days after their inoculation the mycelium reached the edges of the 9 cm Petri plates (Table 2.3.1). They were included in group 1, the fast growing fungi. *F.oxysporum* f.sp. *lactucae* and *P. ultimum* showed slightly slower growth: four days after inoculation, their radial growth was 17.51 mm and 18.10 mm, respectively. Therefore, they were classified as group 2, the medium growing fungi. Lastly, *Phytophtora* sp. and *T. basicola* showed slow growth, four days after the inoculation they showed 10.62 mm and 12.17 mm of radial growth, respectively. Consequently, they were part of group 3, the slowly growing fungi. For the dual culture assays, the antagonist-pathogen distance was set at 25 mm, equal to the

average two-day-growth, for the fungi of group 1, whereas 20 mm and 10 mm, equal to four days of growth for the group 2 and 3, respectively (Table 2.2.1).

Table 2.3.1 – Daily mycelium radial growth for the tested pathogens.

		Mycelium radial growth (mm)						
		day 1	day 2	day 3	day 4	day 5	day 6	day 7
Fungal strain	*F. oxysporum* f.sp. *lactucae*	2.30	8.60	14.00	18.10	21.00	24.10	30.00
	Phytophtora sp.	3.60	6.37	9.12	10.62	9.40	11.28	14.37
	P. ultimum	2.50	8.62	13.03	17.51	21.89	26.27	30.65
	R. solani	10.08	23.58	35.42	*	*	*	*
	S. sclerotiorum	5.87	22.62	37.87	*	*	*	*
	T. basicola	2.63	5.67	8.58	12.17	15.25	18.00	23.50

*: plate full

Antagonist inoculation timing

In dual culture assay, the streptomycete is generally inoculated few days before the pathogen. To find out the optimal timing for interaction between microorganisms, streptomycete reference strains were inoculated at different time points before the pathogen (Table 2.3.2). In the case of *S. sclerotiorum*, the first day of mycelium growth measurement, no significant differences resulted in the inhibition among the trials. In the next two days, when streptomycete strain was inoculated two or three days after the pathogen (trials C and D, respectively), it resulted in significantly stronger inhibition, especially in the case of CMJ57I. Therefore, for the fungi of the Group 1, streptomycete strain was inoculated two days before the pathogens (Table 2.2.1).

For *F. oxysporum* f.sp. *lactucae*, during the first three days of mycelium growth measurement, the streptomycete inoculated the same day of the pathogen (trial A) resulted in significantly lower inhibition compared to the other trials, especially in the case of CMJ57I (Table 2.3.3). In the next days, the inhibition rates were not significantly different. As a consequence, streptomycete and pathogen were inoculated the same day in the case of pathogens of Group 2 and Group 3 (Table 2.2.1).

Table 2.3.2 – Effect of different inoculation timing of *Streptomyces* strains on inhibition of *Sclerotinia sclerotiorum* mycelium growth.

		Trials	**Mycelium growth inhibition (%)**		
			day 1	day 2	day 3
Streptomycete strain	CMJ57I	A	0 a[1]	12.71 b	58.43 b
		B	0 a	31.93 b	67.12 b
		C	20.00 a	56.45 a	77.35 a
		D	10.00 a	63.33 a	80.99 a
	CX14W	A	16.67 a	21.03 a	63.50 b
		B	0 a	39.80 a	71.61 ab
		C	20.00 a	46.47 a	74.65 a
		D	28.57 a	47.22 a	74.57 a

[1] Tukey post-hoc test; mean values in one column with the same letters are not significantly different ($P = 0.05$).

Table 2.3.3 – Effect of different inoculation timing of *Streptomyces* strains on inhibition of *Fusarium oxysporum* f.sp. *lactucae* mycelium growth.

		Trials	**Mycelium growth inhibition (%)**						
			day 1	day 2	day 3	day 4	day 5	day 6	day 7
Streptomycete strain	CMJ57I	A	0 b[1]	0 b	18.18 c	29.41 a	40.91 a	49.07 b	56.06 a
		B	0 b	14.29 ab	25.00 b	38.24 a	44.44 a	55.36 a	56.06 a
		C	0 b	21.43 a	25.00 b	38.89 a	45.65 a	51.72 ab	58.82 a
		D	33.33 a	14.29 ab	30.77 a	38.89 a	43.48 a	51.72 ab	56.06 a
	CX14W	A	50.00 a	14.29 a	35.90 a	42.81 a	48.94 a	55.92 a	59.41 a
		B	16.67 a	21.43 a	25.00 a	36.11 a	43.75 a	48.28 b	55.71 a
		C	50.00 a	28.57 a	32.05 a	36.11 a	44.66 a	51.72 ab	55.88 a
		D	25.00 a	25.00 a	33.33 a	38.24 a	44.47 a	55.36 a	60.29 a

[1] Tukey post-hoc test; mean values in one column with the same letters are not significantly different ($P = 0.05$).

Assessment of mycelium inhibition

Streptomycete and pathogen were inoculated based on the results obtained (Table 2.3.2and Table 2.3.3). The inhibition assessment was set at 2 days after pathogen inoculation for the group 1, 6 and 7 days after inoculation for group 2 and 3, respectively.

Table 2.3.4 - Inhibition of mycelium growth after pathogen inoculation.

			Mycelium growth inhibition (%)						
			day 1	day 2	day 3	day 4	day 5	day 6	day 7
Pathogen group	Group 1	CMJ57I	20	56.41	77.33	/	/	/	/
		CX14W	25.00	46.88	74.65	/	/	/	/
	Group 2	CMJ57I	0	0	18.18	29.41	40.91	49.07	/
		CX14W	50.00	14.29	35.90	42.81	48.91	55.92	/
	Group 3	CMJ57I	0	0	0	30.30	50.00	55.55	62.50
		CX14W	0	0	0	27.27	50.00	55.55	62.50

/ = not determined

2.3.2 Screening of Streptomycete strains for their inhibitory activity against diverse fungal pathogens

Ninety-three % of streptomycetes inibited *Fusarium oxysporum* f.sp. *lactucae* in range from 0.73 to 60% and 52% resulted more active than *Streptomyces lydicus* WYEC 108, which showed 22.46% inhibition. The most active strain was TXB24R. Out of 200 streptomycetes, 193 strains resulted active against *Pythium ultimum* and the most active was LYC01E (68.75%). Thirty-one percent resulted more active than *S. lydicus* WYEC 108, which showed 47.73% inhibition. Twenty-two percent of strains did not inhibit the mycelium growth of *Pytophthora* sp., and 47% resulted more active than *S. lydicus* WYEC 108. The most active strain was AM27W with 76% inhibition activity. Concerning the inhibition against *Rhizoctonia solani*, 74% of streptomycetes had values ranging from 0.94 to 78%. CX16W was the most active strain and the 41% of streptomycetes were more active than *S. lydicus* WYEC 108. *Sclerotinia sclerotiorum* was the pathogen inhibited by most *Streptomyces* strains: only 3 strains out of 200 did not show any activity. Streptomycetes activity ranged from 0.97% to 90.5% and LAU18R was the most active strain. Forty-one percent of strains were more active than *S. lydicus* WYEC 108. Finally, 7 strains out of 200 did not show activity against *Thielaviopsis basicola*. Forty-four percent of strains inhibited the pathogen more than *S. lydicus* WYEC 108 and MR05W resulted the most active strain with 88.10% inhibition activity (Table 2.3.5)

Table 2.3.5 - Inhibition activity of *Streptomyces* strains on mycelium growth of soil borne pathogens.

Strain	Mycelium growth inhibition (%)					
	Fusarium oxysporum* f.sp. *lactucae	***Pythium ultimum***	***Pytophthora* sp.**	***Rhizoctonia solani***	***Sclerotinia sclerotiorum***	***Thielaviopsis basicola***
ALC03R	29,93	41,25	12,00	56,48	65,37	33,33
ALG06R	0,00	18,18	1,85	47,86	37,50	56,67
ALG07R	27,91	57,69	53,70	71,68	75,96	64,00
ALP07R	7,14	20,00	13,56	15,09	86,89	46,43
ALP10R	2,86	4,24	11,02	11,32	27,18	60,71
ALP11R	5,71	38,18	55,56	51,42	53,98	35,71
AM10A	22,5	6,41	5,08	12,96	63,72	48,72
AM12A	23,19	52,94	62,79	44,23	47,62	58,33
AM27W	31,39	59,62	76,27	48,15	48,51	48,72
ARF07R	8,03	5,77	59,32	30,91	19,80	35,90
ARF09R	24,29	57,69	61,82	58,41	68,27	24,00
ARF16R	32,86	56,82	54,72	55,71	60,23	38,46
ARF24R	27,01	47,50	50,00	50,93	68,35	0,00
ARN01R	34,29	34,55	51,85	69,58	56,31	54,76
ARN02R	32,5	39,39	65,25	24,53	35,92	63,10
ARN08R	27,5	24,24	18,52	0,00	27,18	35,71
ARN09R	1,25	38,79	11,11	33,02	13,59	59,52
AST32I	9,42	49,26	9,30	56,14	62,86	45,24
AZ112I	31,39	57,35	30,23	63,11	74,29	45,24
AZ117I	25,36	56,62	30,23	67,48	61,90	51,19
AZ144I	31,88	54,41	44,19	56,31	66,67	50,00
BT08A	0,00	10,26	12,73	33,63	38,46	32,00
BT15W	36,25	54,55	62,26	50,00	57,39	0,00
BT17W	27,14	54,49	60,00	47,22	69,03	70,45
BT28W	6,57	21,25	46,00	20,61	53,85	42,86
BT29W	11,25	9,70	25,93	39,62	42,72	53,33
CHL01R	4,65	38,46	56,36	16,67	56,64	31,82
CM16A	13,18	65,38	58,18	61,84	57,95	58,67
CM20A	4,23	17,50	44,00	32,58	40,00	40,48
CMJ57I	36,23	62,82	62,79	68,93	71,43	76,79
CMJ58I	28,99	57,35	74,42	41,75	46,79	76,19
CMJ60I	23,19	50,00	55,81	50,00	62,39	55,95
CN05W	21,71	27,27	30,19	20,00	28,41	34,62
CN06W	25,71	27,27	52,83	7,02	29,00	35,90
CN08W	36,00	42,50	29,09	42,59	60,55	20,00
CN09W	30,43	33,82	16,28	9,71	40,00	35,71
CN13W	11,63	6,41	3,64	58,77	46,32	28,00
CPB02R	7,5	9,09	16,95	0,94	31,07	60,71
CPB08R	8,00	13,75	29,63	29,21	65,14	28,57

Strain	Mycelium growth inhibition (%)					
	Fusarium oxysporum* f.sp. *lactucae	***Pythium ultimum***	***Pytophthora* sp.**	***Rhizoctonia solani***	***Sclerotinia sclerotiorum***	***Thielaviopsis basicola***
CPB11R	0	37,18	0,00	34,72	62,83	42,22
CR13A	6,57	37,18	10,17	26,36	58,42	40,00
CRC04R	0,00	9,09	38,89	27,03	52,22	52,38
CRM05R	13,18	46,21	56,60	42,86	44,32	43,59
CRM14R	30	19,23	58,14	42,59	73,86	55,56
CRM31R	33,79	0,00	0,00	0,00	23,86	62,22
CSM12R	2,86	13,94	5,56	5,66	9,71	38,10
CU07W	30,23	13,64	64,15	39,05	33,52	46,15
CVM02R	47,5	44,87	66,10	41,67	77,88	64,44
CX08W	0,00	63,64	22,22	50,00	26,70	36,36
CX14W	39,86	61,76	75,58	66,99	78,63	80,36
CX16W	23,26	53,03	73,58	78,07	85,28	66,67
CX17W	31,25	10,00	12,00	25,93	22,73	26,67
EM05W	29,87	49,04	56,36	60,62	64,42	48,00
EP03W	12,5	10,00	14,81	0,00	50,44	0,00
EP05W	10,22	38,13	12,00	25,44	48,57	28,57
EP07W	15,58	51,52	61,82	41,59	54,81	41,33
EP11W	11,59	33,82	12,79	13,59	44,95	21,43
EP17A	40	57,35	74,42	69,30	76,07	80,95
EPH11R	45,45	57,05	61,82	43,36	63,46	58,67
EPH21R	17,5	9,09	0,00	44,44	67,05	43,33
EPH36R	18,25	6,41	8,47	7,27	21,78	46,67
EQS04R	20,93	38,46	58,18	17,70	42,31	38,67
ER18A	14,67	25,00	50,00	46,49	54,29	42,86
ER19A	5,33	9,877	8,00	5,26	49,52	33,33
ER20A	27,13	54,55	5,67	71,43	69,32	65,38
EW13W	45,45	53,85	61,82	42,48	65,38	68,00
EW15W	34,48	0,00	66,04	44,29	55,68	23,08
EW16W	34,74	65,38	63,64	55,75	67,31	56,67
FA01W	13,18	34,85	55,56	42,59	35,80	53,85
FA05W	38,03	46,25	46,00	54,49	54,29	76,19
FA07W	48,05	49,36	56,36	55,75	68,27	78,67
FIC11R	18,25	11,52	18,52	3,64	23,30	46,67
FIC35R	35,77	5,13	0,00	46,36	67,33	33,33
FR05W	13,18	59,62	59,09	59,72	62,50	35,90
FS02W	0,78	53,85	61,82	60,53	61,36	23,30
FS12W	29,87	64,24	65,45	69,47	68,83	50,00
FS18W	26,74	65,432	18,52	72,79	67,78	65,33
FS26W	17,83	59,09	62,26	58,57	59,09	46,15
FT04W	6,57	33,75	50,00	28,07	55,45	38,10
FT05W	35,51	41,03	46,51	68,93	76,92	33,33

Strain	Mycelium growth inhibition (%)					
	Fusarium oxysporum* f.sp. *lactucae	***Pythium ultimum***	***Pytophthora* sp.**	***Rhizoctonia solani***	***Sclerotinia sclerotiorum***	***Thielaviopsis basicola***
FT06W	45,45	55,77	54,55	53,10	66,83	76,00
GC02W	20,16	28,21	58,18	26,99	17,31	41,33
GC03W	6,67	23,75	50,00	42,59	64,10	45,24
GNL02R	23,26	43,18	66,04	53,33	75,76	69,23
HLC02R	2,19	12,82	27,12	3,64	20,45	33,33
HLP01R	28,00	41,25	56,36	55,56	65,14	44,05
HLP03R	32,41	51,52	52,83	53,81	64,20	35,90
HLP08R	0	3,03	5,93	34,91	66,50	45,24
HYP03R	10	30,30	61,11	9,43	0,97	38,89
HYP23R	6,25	29,70	56,78	0,00	0,00	51,11
KAL01R	0,00	14,10	0,00	25,45	65,35	23,08
LAU18R	42,86	48,75	46,00	44,44	90,48	84,62
LM01W	6,49	15,38	58,18	46,02	41,35	65,33
LM07W	31,03	58,33	58,49	56,19	58,24	50,00
LM08W	2,33	6,82	58,49	42,86	29,55	53,85
LMN06R	13,33	2,50	45,45	4,63	39,45	19,23
LMP64I	26,09	48,72	13,95	42,72	51,28	35,71
LMP72I	31,39	35,29	0,00	35,96	17,95	37,50
LMP74I	25,55	45,59	67,44	46,05	54,98	75,00
LRS17R	7,79	7,05	4,55	21,24	13,46	14,67
LRS20R	28,00	15,00	3,64	19,10	41,28	22,62
LRS39R	34,67	46,25	70,91	51,69	72,02	45,24
LRS40R	3,10	24,24	62,96	37,14	26,14	0,00
LRS45R	13,33	16,25	12,00	15,73	6,49	29,76
LT05W	31,03	19,753	51,89	62,28	51,70	84,62
LYC01E	37,33	68,75	62,00	73,15	73,33	73,81
LYC02E	29,33	65,00	62,00	74,07	74,36	69,05
MR01W	27,54	27,94	67,44	18,27	46,15	85,71
MR02W	0,00	44,12	65,12	47,81	44,95	83,33
MR05W	32,85	37,50	58,14	58,77	46,15	88,10
MR11W	0,00	49,24	51,85	47,62	53,98	80,77
MR13W	37,23	45,00	56,00	51,85	54,29	73,81
MR16W	0,00	0,00	16,98	30,95	23,86	40,00
MR19W	0,00	16,67	60,00	44,25	53,85	64,00
MR20W	25,58	51,28	61,82	43,36	61,54	60,00
MR24W	7,75	21,79	61,82	41,59	50,96	68,00
MRX13R	24,64	44,12	65,12	49,12	47,86	72,62
MRX44	16,79	30,88	0,00	17,54	45,30	16,67
NPH11R	31,82	42,31	58,18	46,02	58,65	60,00
OCB07R	24,00	12,00	63,64	43,06	44,04	35,71
OCB10R	27,14	58,75	18,00	46,30	66,23	76,92

Strain	Mycelium growth inhibition (%)					
	Fusarium oxysporum* f.sp. *lactucae	***Pythium ultimum***	***Pytophthora* sp.**	***Rhizoctonia solani***	***Sclerotinia sclerotiorum***	***Thielaviopsis basicola***
OCB15R	29,58	52,56	0,00	35,96	36,70	56,82
OCB21R	29,93	30,77	61,82	40,91	74,75	16,67
PLM01R	55,84	26,92	54,55	58,41	67,31	49,33
PLR02R	0,73	9,62	16,95	18,18	55,94	25,64
PO03W	24,64	32,35	69,77	56,73	60,68	78,57
PO07W	18,67	46,25	50,00	43,82	51,43	34,52
PRT06R	0,00	36,25	50,00	3,60	47,71	19,23
PRT07R	19,77	34,62	52,73	38,10	28,41	36,54
PRV04R	2,5	10,30	30,51	16,98	62,14	53,57
PTH08R	8,03	23,08	22,22	12,73	52,97	74,36
QR03W	6,49	0,00	16,36	30,97	39,42	0,00
QR06W	22,48	9,62	58,18	43,36	35,23	36,00
QR16W	9,86	22,50	64,00	33,71	43,81	61,90
QR19W	3,88	30,30	28,30	27,14	31,82	34,62
QR26W	0,73	55,88	55,81	42,54	46,15	67,86
QR29W	28,00	45,00	52,00	31,48	64,10	38,10
RBF05R	6,49	4,49	12,73	8,85	41,35	24,00
RBF10R	24,09	38,46	66,10	48,18	39,85	54,44
RFB14A	13,18	39,74	22,22	56,58	38,64	60,00
RMX14R	42,07	6,82	52,83	69,52	30,68	61,54
RMX17R	31,43	64,42	61,82	63,16	67,10	52,22
ROS77F	9,33	25,00	10,00	2,78	47,62	30,95
RSM08R	38,75	0,00	55,51	49,06	4,85	64,44
RSM10R	8,57	25,64	52,73	42,98	19,32	38,64
S. lydicus WYEC 108	22,46	47,73	52,00	22,33	56,88	50,00
SG04W	24,03	10,26	60,00	45,13	46,59	78,67
SG06W	17,33	46,25	52,00	43,52	56,41	78,57
SG09W	9,33	0,00	50,00	3,70	0,00	71,43
SG10W	26,81	51,47	66,67	39,42	52,99	85,71
SG12W	28,75	7,88	37,29	30,19	58,25	60,71
SJ01W	17,44	1,28	58,18	37,14	28,41	44,87
SJS02R	15,50	37,12	54,55	39,05	34,09	76,92
SLF27R	47,86	33,94	55,56	72,41	46,90	28,89
SLP02R	25,55	14,81	50,00	27,27	71,29	41,11
SN02A	21,25	4,24	10,17	25,47	51,46	40,48
SPJ01R	41,61	61,54	72,88	72,73	72,77	64,10
ST06W	6,49	12,73	61,82	20,35	40,87	44,00
ST07W	24,81	28,21	20,00	24,78	25,00	28,00
SUA02R	2,19	8,97	16,95	28,18	37,62	0,00
SUA03R	31,03	51,28	60,38	66,67	61,36	34,62
SW01W	31,17	29,49	60,00	38,05	54,81	60,00

Strain	Mycelium growth inhibition (%)					
	Fusarium oxysporum* f.sp. *lactucae	***Pythium ultimum***	***Pytophthora* sp.**	***Rhizoctonia solani***	***Sclerotinia sclerotiorum***	***Thielaviopsis basicola***
SW06W	42,07	53,79	60,38	57,62	90,34	11,54
SW27W	33,77	60,26	61,82	56,64	74,04	41,33
SW29W	38,62	59,09	56,60	68,57	86,93	61,54
SW35W	33,12	48,08	56,36	39,82	52,88	45,33
SW37W	18,60	56,06	37,04	57,14	52,84	43,18
SW42W	7,79	13,46	20,00	21,24	43,27	22,67
SW46W	6,49	17,31	12,73	7,08	20,19	25,33
SYS01R	33,79	15,15	0,00	40,00	28,41	34,62
SYS08R	49,35	53,85	58,18	44,25	59,13	58,67
SYS13R	29,66	34,09	56,60	18,42	47,19	38,46
TAG17R	6,57	5,77	53,70	32,73	58,42	12,22
TOR01L	0,00	50,00	20,00	22,52	73,27	54,76
TOR16L	34,375	11,11	20,34	6,36	13,86	0,00
TOR51L	24,09	7,69	55,56	10,91	39,11	25,64
TOR57I	9,33	30,00	50,00	7,30	44,95	32,14
TOR62L	40,15	37,50	8,00	52,78	72,48	82,05
TOR65I	0,00	47,50	0,00	41,57	58,42	74,36
TRX03R	8,03	19,23	52,54	40,91	32,18	38,46
TXB01R	24,09	4,94	0,00	29,09	40,59	23,08
TXB16R	39,61	68,48	69,09	53,10	68,27	64,44
TXB24R	60,06	44,87	50,91	64,60	71,15	54,67
VLA11R	24,00	32,50	44,00	32,58	47,71	57,14
VLA34R	37,66	52,56	61,82	48,67	58,17	81,33
VNC12R	17,33	41,25	23,64	4,63	18,61	15,38
VRN01R	28,00	13,75	52,00	43,52	45,71	73,81
VT041R	38,67	15,00	38,89	35,96	59,05	9,52
VT098I	25,36	33,82	50,00	54,81	61,54	79,76
VT101I	0,00	20,59	34,00	51,92	56,44	41,67
VT104I	28,99	48,53	40,00	57,69	54,70	51,19
VT105I	21,74	47,79	44,00	59,65	64,96	47,62
VT111I	28,47	55,88	26,00	53,95	64,10	52,38
VT334R	5,80	39,38	48,00	43,52	0,00	61,90
VT394R	5,33	10,00	0,00	0,00	11,43	33,33
VTV06R	3,65	3,85	3,39	14,81	47,52	23,08
YRU27D	43,75	49,38	48,15	41,67	87,50	86,67
YSO07R	40,15	0,00	69,49	50,44	59,41	69,23
ZEA07I	32,85	29,41	0,00	44,74	53,85	36,90
ZEA13I	20,29	54,41	44,00	55,70	59,83	53,57
ZEA17I	34,06	50,74	62,00	67,54	79,49	51,79

2.4 Conclusions

When bacteria are searched as potential Biological Control Agents (BCAs), the dual culture assay often represents the first step (Pliego *et al.*, 2011). Even if the methodology is widely applied and reported in literature, different methods regarding the timing and distances of microorganisms inoculation are described. In this work, a standardized method was proposed. The distance between antagonist and pathogen was set on the basis of the mycelium growth rate of the pathogen to have equal interaction time between the two microorganisms. In some works, streptomycete strain was inoculated before the pathogen to facilitate its growth, sporulation and release of antifungal and antibiotic compounds, since streptomycetes start to produce secondary metabolites when the aerial mycelium is differentiated (Chater and Bibb, 1997). Our results showed that when the antagonist was inoculated before the pathogen it generally inhibited mycelium growth to higher extent than the same day inoculation during the first days of measurement, however, in the next days the differences were not significant among different inoculation days.

The parameters studied in this work were standardized for the growing condition of these assays: the CZY and PDA media and 24°C as incubation temperature, and need to be adapted for different cultivation conditions. It is well known, that the incubation temperature and type of medium can influence the mycelium growth of the fungi. Often, the mycelium grows slower at low temperatures than at high temperatures. Therefore, the proposed methodology should be verified and adapted before the preparation of every dual culture assay according to the applied growing conditions.

Once the method of dual culture was optimized, a collection of streptomycete strains was screened for the antagonistic activity against six soil borne fungi (Figure 2.4.1). Almost all streptomycete stains inhibited the pathogens and many of them were more active than the reference strain *S. lydicus* WYEC 108. Their activity was variable for different pathogens: some of them resulted strongly active against one fungus but not against the others. For instance, LAU18R was the most active against *S. sclerotiorum*, but showed little activity against the other pathogens. This suggests that different and specific modes of action are involved in the antagonism and further studies are needed, that would identify array of secondary metabolites released by promising strains involved in the antifungal activity against diverse fungal pathogens. However, it is important to highlight that the dual culture assays represent only the first step towards the identification of effective BCAs. For antagonists that act at root level, the rhizosphere competence is an important prerequisite for obtaining successful biocontrol (Compant *et al.*, 2010). Therefore, the ability of promising strains to survive in soil, colonize the rhizosphere and the roots was the next step studied for a successful development of a BCAs. Finally, the efficacy of the most active *Streptomyces* strains must be

investigated *in planta*. In the following chapters the experiments in peat soil and in natural soil both under controlled conditions and in field are described.

Figure 2.4.1 – Antagonistic activity of *Streptomyces* spp. SW06W, SW29W and SYS08R against *Sclerotinia sclerotiorum.*

3 Chapter 3: Screening of *Streptomyces* strains with Plant Growth Promoting (PGP) activity

3.1 Introduction

The rhizosphere is the part of soil that surrounds and is influenced by plant roots (Philippot *et al.*, 2013). It is defined an "hot spot" in the soil due to numerous interactions which occur among the microorganisms that inhabit the rhizosphere, the plant and the exudates released by roots. Root exudates include amino acids, organic acids, phenolics, polysaccharides and proteins and play a fundamental role in the rhizosphere. Many microorganisms are chemoattracted by nutrient exudates, a phenomenon known as "rhizosphere effect" (Bais *et al.*, 2006). Once in the rhizosphere, the organisms can have a neutral or deleterious effect on the plant growth and health, or support their hosts and promote their development (Berg, 2009; Lugtenberg and Kamilova, 2009; Raaijmakers *et al.*, 2009). The bacteria able to exert a beneficial effect to plant growth are termed Plant Growth Promoting Bacteria (PGPB, (Bashan and Holguin, 1998)) or Plant Growth Promoting Rhizobacteria (PGPR, (Kloepper and Schroth, 1978)). In the last years, extensive researches have demonstrated that PGPB can have an important role in agriculture and horticulture in improving crop productivity (Lucy *et al.*, 2004; Vessey, 2003). Many isolates have been studied as beneficial bacteria for crop production and stimulation of different crops by PGPB has been demonstrated. For instance, the inoculation of auxin-producing bacteria caused the increase in growth and yield of wheat (Khalid *et al.*, 2004), as well as root and shoot elongation was recorded in seedling of different crop species inoculated with a PGP-*Pseudomonas putida* (Hall *et al.*, 1996). Genera of PGPR include *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Acetobacter*, *Burkholderia*, *Bacillus*, *Paenibacillus* (Raj *et al.*, 2006). The PGPB have numerous modes of action. Generally, they increase the availability of nutrients for the plant in the rhizosphere. Iron is an essential nutrient for plants, and roots absorb it as the ferrous (Fe^{2+}) ion. However, the ferric (Fe^{3+}) ion is more commonly found in soil. Some PGPB produce siderophores (Greek: iron carrier), which bind Fe^{3+}. These chelators "deliver" the ferric ion to the root surface where it is reduced to ferrous ion and immediately absorbed by plant (Neilands, 1995). Furthermore, PGPB can improve the availability of phosphorus for plant nutrition. Phosphorus is one of the essential macronutrients for plant growth and development, but in soil only a tiny amount is available for plants. The low availability of phosphorous to plants is because the vast majority of soil phosphorus is found in insoluble forms, and plants can only absorb this element in two soluble forms, the monobasic ($H_2PO_4^-$) and the dibasic (HPO_4^{2-}) ions. Phosphate solubilizing bacteria can secrete organic acids and phosphatases facilitating the conversion of insoluble forms of phosphorus to plant-available forms (Rodrìguez

and Fraga, 1999). PGPB can also produce or change the concentration of hormones (auxins, cytokinin, gibberellins and ethylene), which regulate plant development (Glick, 1995; Vessey, 2003). The phytohormone indoe-3-acetic acid (IAA) is one of the most important auxins. It is involved in root initiation, cell division and cell enlargement. Most commonly, IAA-producing PGP bacteria are known to increase root growth and length, resulting in greater root surface area which enables the plant to access more nutrients from soil (Duca *et al.*, 2014; Vessey, 2003). The amino acid tryptophan, which is released by roots, serves as precursor for biosynthesis of auxins in plants and microbes (Sarwar *et al.*, 1992; Spaepen *et al.*, 2007). Moreover, rhizosphere bacteria enhance plant tolerance against salt and drought stresses (Yang *et al.*, 2009). Under stress conditions, the hormone ethylene is endogenously produced by plant, resulting in reduced plant growth. The bacterial enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase degrades the ethylene precursor ACC, releasing plant stress and restoring normal plant growth (Glick *et al.*, 2007).

Also members of the genus *Streptomyces* have been reported for the PGP activity (de Vasconcellos and Cardoso, 2009; Sousa *et al.*, 2008). Especially, they are known to synthesize the hormone auxin (Manulis *et al.*, 1994) and improve the availability of iron (Imbert *et al.*, 1995) and phosphate in the rhizosphere (Sousa *et al.*, 2008).

The goal of this work was to study some traits involved in the PGP activity of 200 strains of the genus *Streptomyces*, specifically, the synthesis of IAA, the production of siderophores and the phosphate solubilization.

3.2 Methodology

3.2.1 *Streptomyces* culture

A collection of 200 streptomycete strains was used in the experiments. They were stored at -20°C and inoculated as spore suspension (see Paragraph 1.2 for details).

3.2.2 Indole-3-acetic acid production

Indole-3-acetic acid (IAA) production was evaluated according to the method proposed by Bric *et al*. (Bric *et al.*, 1991). Tubes were prepared with 5 mL of Czapek broth added with 500 µg/mL of L-tryptophan (Sigma-Aldrich-USA) and were inoculated with 10 µL of streptomycete spore suspension. Three replicates for each strain were prepared. Tubes were incubated at 26°C with constant shaking at 125 rpm in the dark. After 10 days, 2 mL of grown culture were centrifuged at 13000 rpm for 10 minutes. Two mL of supernatant were mixed with 100 µL of 10 mM orthophosphoric acid and 4 mL of Salkowski reagent. After 20 minutes of incubation at room temperature, the development of pink color indicated the IAA production. The optical density (OD)

was measured with the spectrophotometer at 530 nm. The concentration of IAA produced by the strains was calculated with the help of standard curve of IAA (Sigma-Aldrich,USA) obtained in the range of 1-30 µg/mL.

3.2.3 Siderophore production

Streptomycetes were evaluated for siderophore production according to Pérez-Miranda *et al*. (Pérez-Miranda *et al.*, 2007). Ten µL of agar-spore suspension (10 µL of spore suspension in 90 µL of 0.2% water agar) were inoculated in the middle of a Petri plate (90 mm diameter) containing modified Czapek medium for siderophores production. Plates were incubated for 14 days at 24°C. Subsequently, 15 mL of Chrome Azurol S agar were cast upon culture agar plates. After 1 day of incubation at room temperature in the dark, the change of color around the colony (from blue to orange) indicated the siderophore production. The orange halo (D) and colony (d) diameters were measured and the production of siderophores was expressed as (D -d)/2.

3.2.4 Phosphate solubilization

The NBRIY medium devoid of yeast extract was used to assess the phosphate solubilization of streptomycete strains. The pH of the medium was adjusted to 7.0 before autoclaving. Ten µL of agar-spore suspension (10 µL of spore suspension in 90 µL of 0.2% water agar) were inoculated in the middle of a Petri plate (90 mm diameter) and 3 replicates were prepared for each strain. Plates were incubated at 24°C for 14 days in the dark. The halo (D) and the colony (d) diameters were measured and the phosphate solubilization ability was expressed as (D – d)/2.

3.3 Results

The production of IAA is a common PGP trait among the isolates (Table 3.3.1). Only six strains did not synthesize auxins. The other strains produced IAA with amount that ranged from 0.05 to 22 µg-mL (Table 3.3.1). Strains AM12A, CN08W and RSM10R showed the highest IAA production (20-22 µg/mL).

Only 22% of streptomycete strains showed the production of siderophores. The radius of the halo ranged from 5 to 40 mm and HLP01R and ST07W were strains that showed the widest halo of siderophore production on CAS agar.

Finally, only 18.5% of isolates showed the ability to solubilize phosphate in NBRIY medium. For these strains, the clear halo around the colony was very thin: only 1 mm.

Considering altogether the traits that can contribute to the PGP activity, 15 strains out of 200 showed the ability to produce IAA, siderophores and the P solubilization. Among them, ALC03R and BT08A were the most active.

Table 3.3.1– Indole-3-acetic acid and siderophore production and phosphate solubilization activity of 200 streptomycete strains. (+: halo width 1 mm; -: not active).

Strain	Indole-3-acetic acid (µg-mL)	Siderophore (mm)	Phosphate solubil. activity	Strain	Indole-3-acetic acid (µg-mL)	Siderophore (mm)	Phosphate solubil. activity
ALC03R	5.78	7.10	+	CN09W	3.36	-	-
ALG06R	7.24	9.00	-	CN13W	4.40	-	-
ALG07R	1.88	-	-	CPB02R	0.49	-	-
ALP07R	0.00	-	+	CPB08R	1.31	-	-
ALP10R	1.73	-	-	CPB11R	1.91	-	+
ALP11R	2.38	-	-	CR13A	0.21	-	-
AM10A	0.62	7.25	-	CRC04R	0.02	6.88	-
AM12A	22.24	-	-	CRM05R	4.57	-	-
AM27W	0.77	-	-	CRM14R	19.45	-	+
ARF07R	4.31	-	-	CRM31R	16.48	-	-
ARF09R	1.01	25.1	-	CSM12R	1.16	-	-
ARF16R	2.91	-	-	CU07W	1.45	-	-
ARF24R	1.45	7.3	-	CVM02R	4.67	29.13	+
ARN01R	1.72	-	-	CX08W	1.06	12.70	-
ARN02R	3.46	-	-	CX14W	3.13	-	-
ARN08R	2.96	-	+	CX16W	1.25	10.95	+
ARN09R	7.24	-	-	CX17W	15.52	10.90	-
AST32I	4.13	-	-	EM05W	1.92	-	-
AZ112I	3.87	-	-	EP03W	1.39	-	-
AZ117I	3.79	-	-	EP05W	0.90	-	-
AZ144I	4.11	-	-	EP07W	15.15	-	-
BT08A	6.51	33.25	+	EP11W	4.06	-	-
BT15W	2.47	-	-	EP17A	3.77	22.00	+
BT17W	0.17	-	-	EPH11R	0.00	-	-
BT28W	1.06	-	-	EPH21R	0.65	6.63	-
BT29W	4.49	-	-	EPH36R	4.79	-	-
CHL01R	0.86	-	-	EQS04R	2.12	-	+
CM16A	3.94	-	-	ER18A	1.57	-	+
CM20A	2.07	-	+	ER19A	0.96	12.70	-
CMJ57I	2.67	-	-	ER20A	3.48	-	-
CMJ58I	4.25	-	-	EW13W	2.22	-	-
CMJ60I	5.50	-	-	EW15W	8.07	-	-
CN05W	3.45	-	-	EW16W	8.19	-	-
CN06W	2.73	-	-	FA01W	1.62	-	-
CN08W	22.03	16.10	-	FA05W	3.22	-	-

Strain	Indole-3-acetic acid (µg-mL)	Siderophore (mm)	Phosphate solubil. activity	Strain	Indole-3-acetic acid (µg-mL)	Siderophore (mm)	Phosphate solubil. activity
FA07W	0.00	-	-	MR01W	3.00	-	-
FIC11R	0.29	-	-	MR02W	2.51	-	-
FIC35R	0.88	-	-	MR05W	3.23	12.30	-
FR05W	7.99	-	-	MR11W	1.73	-	-
FS02W	0.75	6.70	-	MR13W	1.71	-	-
FS12W	4.36	-	-	MR16W	3.07	-	+
FS18W	2.22	-	-	MR19W	0.70	-	-
FS26W	8.91	-	-	MR20W	0.95	-	-
FT04W	0.39	-	-	MR24W	2.56	-	-
FT05W	3.52	-	-	MRX13R	2.62	-	-
FT06W	1.95	-	-	MRX44	2.30	-	-
GC02W	0.91	-	+	NPH11R	3.46	-	-
GC03W	1.73	6.50	+	OCB07R	2.64	-	-
GNL02R	2.51	-	-	OCB10R	1.31	6.80	-
HLC02R	13.76	-	-	OCB15R	0.39	-	-
HLP01R	1.64	40.00	-	OCB21R	0.23	-	+
HLP03R	0.48	-	-	PLM01R	1.61	-	-
HLP08R	0.24	-	-	PLR02R	0.39	12.88	+
HYP03R	0.89	-	+	PO03W	3.48	14.00	-
HYP23R	1.03	21.88	+	PO07W	1.08	9.40	-
KAL01R	0.29	-	-	PRT06R	14.27	-	-
LAU18R	8.06	-	-	PRT07R	1.36	-	-
LM01W	1.61	-	-	PRV04R	0.09	9.00	+
LM07W	5.28	-	-	PTH08R	0.62	-	-
LM08W	2.36	-	-	QR03W	0.00	-	-
LMN06R	0.06	24.80	+	QR06W	1.71	9.30	-
LMP64I	1.53	-	-	QR16W	3.26	-	+
LMP72I	3.68	-	+	QR19W	1.81	-	-
LMP74I	2.85	-	-	QR26W	2.62	-	+
LRS17R	3.90	33.25	-	QR29W	1.20	-	-
LRS20R	1.81	14.30	-	RBF05R	6.93	33.50	+
LRS39R	3.38	-	-	RBF10R	3.46	8.90	-
LRS40R	1.49	-	-	RFB14A	8.63	-	-
LRS45R	1.54	-	-	RMX14R	3.30	-	-
LT05W	4.81	-	+	RMX17R	2.55	-	+
LYC01E	2.41	8.90	+	ROS77F	0.86	-	+
LYC02E	4.02	-	-	RSM08R	4.19	19.63	+

Strain	Indole-3-acetic acid (µg-mL)	Siderophore (mm)	Phosphate solubil. activity	Strain	Indole-3-acetic acid (µg-mL)	Siderophore (mm)	Phosphate solubil. activity
RSM10R	20.06	31.00	-	TOR01L	1.85	-	-
SG04W	4.25	-	-	TOR16L	8.21	-	-
SG06W	0.51	-	-	TOR51L	0.73	-	-
SG09W	1.58	-	-	TOR57I	2.50	-	-
SG10W	2.14	-	-	TOR62L	2.34	-	-
SG12W	0.17	9.13	+	TOR65I	1.68	-	-
SJ01W	1.52	-	-	TRX03R	0.90	-	-
SJS02R	6.28	-	-	TXB01R	5.87	-	-
SLF27R	3.01	-	-	TXB16R	0.57	26.00	-
SLP02R	2.32	-	-	TXB24R	5.17	-	-
SN02A	0.76	5.00		VLA11R	4.50	-	+
SPJ01R	2.38	-	-	VLA334R	0.14	1.38	+
ST06W	0.69	2.88	+	VNC12R	3.32	-	-
ST07W	4.09	40.00	-	VRN01R	0.52	-	-
SUA02R	1.34	-	-	VT041R	1.31	-	-
SUA03R	6.79	-	-	VT098I	2.36	-	-
SW01W	3.21	-	-	VT101I	1.63	18.50	-
SW06W	1.88	-	+	VT104I	4.15	-	-
SW27W	2.84	-	-	VT105I	2.88	-	-
SW29W	0.75	8.700	-	VT111I	3.71	-	-
SW35W	1.61	-	-	VT334R	1.59	16.75	-
SW37W	2.91	-	-	VT394R	3.06	-	-
SW42W	1.63	-	-	VTV06R	0.35	-	+
SW46W	0.00	32.20	-	YRU27D	2.70	-	-
SYS01R	4.41	-	-	YSO07R	0.87	-	-
SYS08R	0.00	-	-	ZEA07I	2.91	-	-
SYS13R	1.19	-	-	ZEA13I	3.54	-	-
TAG17R	0.20	-	+	ZEA17I	3.26	-	-

3.4 Conclusions

Bacteria that inhabit the rhizosphere cause neutral, deleterious or beneficial effects on plant growth and heath (Lugtenberg and Kamilova, 2009). Among these, the bacteria that improve plant growth are defined Plant Growth Promoting Bacteria (PGPB) (Bashan and Holguin, 1998).

Bacteria of the genus *Streptomyces* are common inhabitants of rhizosphere and act as beneficial microorganisms for plant growth and development (Gopalakrishnan *et al.*, 2013; Tokala *et al.*,

2002a). The main modes of action involved in the PGP activity are the synthesis of the hormone indole-3-acetic acid (IAA) and the improvement of iron and phosphate availability for the plant in the rhizosphere. Here, a collection of 200 endophytic streptomycetes was analyzed for these PGP traits. IAA was produced by almost all isolates, as commonly reported for bacteria, which inhabit the rhizosphere (Patten and Glick, 1996). The IAA values obtained in this study are in accordance with other IAA producing-streptomycete strains (Abd-Alla *et al.*, 2013; Khamna *et al.*, 2009; Ruanpanun *et al.*, 2010). Strains AM12A, CN08W and RSM10R that showed high IAA values, could be further analyzed for their possible effect on root growth and development. Only few streptomycetes showed siderophore production (22%), among them, strains HLP01R and ST07W produced the widest orange halo indicating synthesis of siderophores (Figure 3.4.1). It is well known that microbial siderophores play an important role in plant growth as demonstrated by the effect on root and shoot biomass and length of rice plants, as consequence of the inoculation of a siderophore-producing streptomycete (Rungin *et al.*, 2012). Finally, few *Streptomyces* strains showed the ability to solubilize phosphate.

Fifteen streptomycete strains which showed multiple PGP activities could be further investigated *in vivo* conditions to study their possible role in promoting plant growth.

Figure 3.4.1 – The production of siderophores by *Streptomyces* spp. CX08W, LMN06R and. RSM08R.

4 Chapter 4: *In vivo* studies on *Streptomyces* strains as BCAs against lettuce drop

4.1 Introduction

In Italy, lettuce (*Lactuca sativa* L.) is marketed for "ready to eat" salads and fresh products and is cultivated under plastic tunnels or in open field. The intensive use of soil and the absence of crop rotation have resulted in an increase of phytopathological problems. especially those caused by soil borne fungi (Gilardi *et al.*, 2010; Gullino *et al.*, 2007).

Among the pathogens of lettuce, *Sclerotinia sclerotiorum* (Lib.) de Bary is one of most important and causes significant yield and economic losses. Mean temperatures around 15°C, high relative humidity and soil water content create optimal environmental conditions for the development of the disease. Symptoms are commonly observed early after emergence, with water-soaked areas on the stem of seedlings near the soil surface. The pathogen colonizes the petioles and subsequently leaves wither and droop lying on the ground. Collapsed leaves are covered with a snowy white mycelium with black sclerotia. Sclerotia represent the survival structures of the pathogen and remain viable in soil for up to 8 years. They play a major role in the disease cycle as a source of primary inoculum (Adams and Ayers, 1979; Bolton *et al.*, 2006). Lettuce drop integrated management strategies are only partially applicable in the Italian horticultural scenario (Barrière *et al.*, 2014). Crop rotation is rare and *S. sclerotiorum* has a wild range of plant hosts (Boland and Hall, 1994; Garibaldi *et al.*, 2005b). Soil solarization is an efficient method to reduce *Sclerotinia* inoculum (Phillips, 1990) but it is partially applicable because the climate would impose too long absence of crop in field. Moreover, the application of soil fumigants is rather restricted as consequence of the last European Directives (Colla *et al.*, 2012). In this scenario, biological control represents a valuable alternative. Biological Control Agents (BCAs) active against *S. sclerotiorum* and available for commercial application in are *Coniothyrium minitans* (strain CON/M/91-08, ContansWG, Intrachem Bio Italia) in Italy and *Streptomyces lydicus* (strain WYEC108, Actinovate, Natural Industries) on the USA market.

This work aimed to study the activity of ten promising *Streptomyces* strains to control lettuce drop.

4.2 The survival analysis

In following experiments the dynamic of lettuce drop incidence was analyzed by the survival analysis method (Kleinbaum and Klein, 2012).

The survival analysis is a family of statistical techniques commonly used in medical studies and engineering under the name of failure analysis. It examines the time it takes an event to occur as outcome variable, which can be the death of a subject, in our case the plant. For example, after a

treatment exposure, the researcher can be interested to determine the time that individual survives after treatment (survival time) for each individual of a population that is followed over a certain period. These procedures rely on two related functions. One of these is the survivor function, denoted as S(t), which is the probability of an individual to survive until a specified time t (event does not occur). Another way to describe survival data is the function denoted by h(t), the hazard function which, in contrast, is the instantaneous potential of that event to occur at time t, given survival up to t. S(t) is a probability and its values range between 0 and 1. In contrast, h(t) is not a probability but, depending on time considered, its value can vary between 0 and ∞. Once one of the two is known, the other can be computed using the formulas:

$$S(t) = exp\left[-\int_0^t h(u)du\right]$$

$$h(t) = \left[\frac{dS(t)/dt}{S(t)}\right]$$

In survival analysis, one of the goals is to compare groups of individuals submitted to different treatments. One way to achieve this goal is, first, to construct survival curves via Kaplan Meier method (KM) and then, to obtain statistical inference on differences between the curves. The KM is a non-parametric estimation of the survival function as it is empirically determined. Suppose that we are following k individuals for whom a certain event occurs at distinct time. Since events are assumed to occur independently, the probability of surviving from one interval to the next are multiplied together to obtain the cumulative survival ($\hat{S}$) by the formula:

$$\hat{S} = \hat{S}(t_{(f-1)}) * \hat{P}\mathrm{r}(T > t_{(f)} \mid T \geq t_{(f)})$$

In particular, it is computed by multiplying the survival estimate for the previous failure time (f-1) by the conditional probability of surviving past the current failure time (f). Between each time of event, the S(t) function is constant and KM curves are plotted as a step function.

One of the most common tests to compare two, or more, survival curves is the logrank test, which gives the χ^2 value calculated as follows:

$$\chi^2 = \sum_i^g \frac{(O_i - E_i)2}{E_i}$$

with *g* equal to the number of groups, O_i to the observed number of events and E_i to the number of events one would expect since the previous event if there were no differences between the groups.
The obtained value is then compared to a χ^2 distribution with g-1 degrees of freedom to compute a final probability of statistical significance for the differences between the groups.
Despite the large use of logrank test in survival analysis, it has more than one limitation: it does not take into account the possible effect of covariates and is not informative about effect size. For this reason, multivariate models approaches have been proposed to analyze survival data. One of the most used was proposed by Cox (Cox, 1972). As other regression models, it specifies explanatory variables (X) and gives the hazard at time t for an individual, using the following formula:

$$h(t, X) = h_0(t)e^{\sum_{i=1}^{p} \beta_i X_i}$$

where: X_i are the explanatory variables and $ß_i$ are the coefficients for each variable included in the model.
One of the Cox model features is that only $h_0(t)$, the baseline hazard function, is time-dependent, whereas the Xs aren't. Moreover, it is a semi-parametric model, because the $h_0(t)$ is not specified. The Cox method has important assumptions: the proportionality of the hazards and the independence of covariates from the time have both to be satisfied. Moreover, using the formula of the Cox model it is possible to compute the point effect of one treatment versus another (or versus a control) the hazard ratio (HR), as follows:

$$\widehat{HR} = \frac{\hat{h}_0(t)e^{\sum_{i=1}^{p} \hat{\beta}_i X^*{}_i}}{\hat{h}_0(t)e^{\sum_{i=1}^{p} \hat{\beta}_i X_i}}$$

simplified as:

$$\widehat{HR} = exp\left[\sum_{i=1}^{p} \beta_{i(X_i^* - X_i)}\right]$$

where X* is the set of predictors for one individual, generally the group with the larger hazard, and X for the other (group with the smaller hazard).

The analyses were performed with R software, version R3.0.2 (R_Core_Team, 2013) and the Package for survival analysis (Therneau, 2014).

4.3 Experiment in growth chamber

4.3.1 Material and methods

The experiment was carried out in the growth chamber (24°C, 55% RH and 15 h photoperiod) and plants (*Lactuca sativa* Batavia Dorè, La Semiorto Sementi, Italy) were sown in plastic boxes (10x10x10 cm) filled with 200 g of growing substrate (Vigorplant, Piacenza, Italy). Plants were watered every 2-3 days with tap water. Three pots were prepared as replicates for each trial. In each box, thirty lettuce seeds were sowed in 3 rows.

Antagonists and soil treatment

Ten *Streptomyces* strains were used in this experiment. They were selected based on their high activity in dual culture assay against *Sclerotinia sclerotiorum* ranging from 75% to 90% (see Chapter 2 for details). Contans (Intrachem Bio, Italy) and Actinovate (Natural Industries, Inc., USA) were used as treatment controls (see Paragraph 1.3 for details). Streptomycete strains were stored as spore suspension in 10% glycerol at -20°C. Streptomycete spore suspensions (10^6CFU/m^2 and 10^8CFU/m^2), Actinovate (1 kg/ha and 100 kg/ha), and Contans (6 kg/ha) were sprayed on top of the growing substrate inoculated with *S. sclerotiorum*.

Preparation of the pathogen inoculum

Twenty-five grams of wheat kernels were sterilized (20 minutes at 121°C) with 50 mL of distilled water in a 300 mL flask. Subsequently, they were inoculated with 10 agar-mycelium discs (6 mm diameter) taken from the edge of an actively growing *S. sclerotiorum* colony grown for three days at 20°C on MEA medium (Budge and Whipps, 2001). Flask was incubated for three weeks at 20°C and was shaken every day to obtain a uniform inoculum. Then, all colonized wheat kernels were blended with 100 mL of sterilized water with a blender to obtain a slurry.

Preparation of the experiment

The growing substrate was added with the *S. sclerotiorum* slurry (1 g of slurry in 100 g substrate). The slurry was dissolved in an adequate volume of water to facilitate the uniform distribution of the inoculum in the substrate. After the inoculation of the growing substrate with the pathogen, the antagonists were applied. Fourteen trials were prepared:

1. Un-inoculated control - growing substrate no inoculated
2. Inoculated control - growing substrate inoculated with *S. sclerotiorum*
3. Contans - growing substrate inoculated with *S. sclerotiorum* and Contans
4. *Streptomyces* ALP07R - growing substrate inoculated with *S. sclerotiorum* and *Streptomyces* ALP07R
5. *Streptomyces* CVM02R - growing substrate inoculated with *S. sclerotiorum* and *Streptomyces* CVM02R
6. *Streptomyces* CX14W - growing substrate inoculated with *S. sclerotiorum* and *Streptomyces* CX14W
7. *Streptomyces* CX16W - growing substrate inoculated with *S. sclerotiorum* and *Streptomyces* CX16W
8. *Streptomyces* FT05W - growing substrate inoculated with *S. sclerotiorum* and *Streptomyces* FT05W
9. *Streptomyces* LAU18R - growing substrate inoculated with *S. sclerotiorum* and *Streptomyces* LAU18R
10. *Streptomyces* SW06W - growing substrate inoculated with *S. sclerotiorum* and *Streptomyces* SW06W
11. *Streptomyces* SW29W - growing substrate inoculated with *S. sclerotiorum* and *Streptomyces* SW29W
12. *Streptomyces* YRU27D - growing substrate inoculated with *S. sclerotiorum* and *Streptomyces* YRU27D
13. *Streptomyces* ZEA17I - growing substrate inoculated with *S. sclerotiorum* and *Streptomyces* ZEA17I
14. Actinovate - growing substrate inoculated with *S. sclerotiorum* and Actinovate

Two variations of the experiment were done on the basis of lettuce sowing times. In the experiment A lettuce was sown the same day of substrate inoculation. In the experiment B lettuce was sown 1 week after substrate inoculation with the competitor and the pathogen. Dead plants were counted from the emergence, which occurred 4 days after sowing, for 18 days in the experiment A and 24

days for the experiment B. The number of dead plants in the three replicates was summed and the disease incidence was calculated as percent of dead plants over the plants germinated in the un-inoculated control.

Statistical analysis

The Hazard Ratio (HR) was calculated as ratio between the hazard function for the trial treated with the antagonist and the inoculated control.

4.3.2 Results

In the experiment A, the lettuce drop incidence in the inoculated control was 27% four days after sowing and reached 86% at the end of the experiment (Table 4.3.1). Significant differences were observed among the trials (χ^2 = 59.2, P = 5.03e-05). There were not significant differences between the two concentrations applied, with exception for *Streptomyces* SW06W and *Streptomyces* YRU27D (Table 4.3.2). The application of Contans, Actinovate, *Streptomyces* CX16W, *Streptomyces* FT05W, *Streptomyces* LAU18R, *Streptomyces* SW06W, and *Streptomyces* YRU27D caused higher, but not significant, risk of disease incidence compared to the inoculated control (Table 4.3.1). In these trials the HR resulted higher than 1 and varied from 1 for *Streptomyces* CX16W to 1.37 *Streptomyces* YRU27D, applied at the higher spore concentration. (Table 4.3.2) which means that the disease incidence was similar to the inoculated control for the former strain, and 37% higher for the latter strain. Only in two trials *Streptomyces* SW06W and *Streptomyces* YRU27D, when the antagonists were applied at lower spore concentration, the HR were significantly higher than 1 (P = 0.04 and P = 0.001, respectively) (Table 4.3.2). In particular, they caused 41% and 73% higher risk of lettuce drop compared to the inoculated control. In few trials, the application of antagonists, for instance *Streptomyces* ZEA17I and *Streptomyces* SW29W, protected the plants against the pathogen (HR < 1), but the protection was not significant (Table 4.3.2)

In the experiment B, the lettuce drop incidence was 38.95% lower in the inoculated control than the inoculated control in the experiment A (χ^2 = 22.3, P = 7.33e-06) (Figure 4.3.1). The disease incidence was 26% four days after the sowing and reached 76% at the end of the experiment (Table 4.3.3). The application of antagonists resulted in significantly different dynamics of disease among trials (χ^2 = 128, P = 1.11e-16) Moreover, also the spore concentration significantly influenced the dynamics of the disease within trails (Table 4.3.4). In few cases, the streptomycete strains caused slightly higher disease incidence (HR > 1, P >0.05). Only inoculation of *Streptomyces* ZEA17I and *Streptomyces* CX16W at either concentration did not have effect on the lettuce drop (P > 0.05)

(Table 4.3.4), whereas the other antagonists significantly reduced disease incidence at least at one concentration applied (Table 4.3.3). For these strains, the HR resulted lower than 1 and HR values varied from 0.34 for Contans to 0.57 for *Streptomyces* YRU27D applied at the lower dose (P = 1.28e-05 and P = 0.011, respectively) (Table 4.3.1). It meant 66% and 43% of protection by these antagonists.

Table 4.3.1 – Disease incidence on lettuce "Batavia" in growth chamber in the experiment A.

Trials			**Disease incidence (%)**								
		Days[1]	*4*	*7*	*8*	*9*	*10*	*11*	*14*	*16*	*18*
	Inoculated control		26.92	60.26	62.82	67.95	70.51	74.36	80.77	85.90	85.90
	Contans		24.36	57.69	67.95	70.51	79.49	83.33	87.18	87.18	88.46
		(CFU/m^2)[2]									
	Streptomyces ALP07R	10^6	21.79	67.95	75.64	82.05	84.62	84.62	85.90	85.90	87.18
		10^8	20.51	52.56	65.38	65.38	67.95	67.95	74.36	75.64	83.33
	Streptomyces CVM02R	10^6	24.36	73.08	79.49	84.62	85.90	87.18	91.03	91.03	92.31
		10^8	20.51	60.26	66.67	71.79	75.64	76.92	78.21	79.49	82.05
	Streptomyces CX14W	10^6	26.92	60.26	73.08	75.64	75.64	79.49	82.05	83.33	83.33
		10^8	34.62	70.51	78.21	79.49	84.62	84.62	88.46	88.46	89.74
	Streptomyces CX16W	10^6	26.92	74.36	85.90	88.46	89.74	91.03	92.31	93.59	94.87
		10^8	28.21	66.67	75.64	80.77	82.05	84.62	84.62	84.62	85.90
	Streptomyces FT05W	10^6	25.64	62.82	71.79	84.62	85.90	88.46	93.59	93.59	92.31
		10^8	24.36	62.82	71.79	75.64	79.49	79.49	80.77	80.77	80.77
	Streptomyces LAU18R	10^6	23.08	71.79	79.49	79.49	82.05	83.33	85.90	87.18	88.46
		10^8	24.36	78.21	79.49	84.62	88.46	88.46	88.46	89.74	89.74
	Streptomyces SW06W	10^6	30.77	73.08	82.05	84.62	88.46	89.74	94.87	94.87	96.15
		10^8	30.77	65.38	74.36	79.49	84.62	87.18	88.46	88.46	89.74
	Streptomyces SW29W	10^6	7.69	46.15	64.10	69.23	74.36	75.64	82.05	84.62	87.18
		10^8	8.97	48.72	65.38	64.10	66.67	70.51	75.64	79.49	80.77
	Streptomyces YRU27D	10^6	34.62	84.62	88.46	89.74	91.03	92.31	93.59	94.87	94.87
		10^8	34.62	74.36	80.77	83.33	84.62	84.62	88.46	87.18	89.74
	Streptomyces ZEA17I	10^6	23.08	61.54	67.95	70.51	71.79	75.64	74.36	74.36	74.36
		10^8	19.23	53.85	74.36	76.92	78.21	80.77	82.05	80.77	84.62
	Actinovate	10^6	17.95	69.23	83.33	84.62	85.90	85.90	87.18	88.46	89.74
		10^8	30.77	73.08	79.49	82.05	82.05	85.90	87.18	87.18	88.46

[1] days after sowing, [2] spore concentration

Table 4.3.2 – The hazard ratio and confidence intervals from the Cox model for lettuce "Batavia" in growth chamber in the experiment A.

			Hazard Ratio (HR)	**Lower 0.95**	**Upper 0.95**	**P**
Trials	Contans		1.035	0.740	1.451	0.840
		(CFU/m^2)[1]				
	Streptomyces ALP07R	10^6	1.147	0.818	1.607	0.426
		10^8	0.902	0.641	1.268	0.552
	Streptomyces CVM02R	10^6	1.189	0.850	1.663	0.313
		10^8	0.925	0.656	1.305	0.658
	Streptomyces CX14W	10^6	0.928	0.660	1.303	0.665
		10^8	1.247	0.888	1.752	0.203
	Streptomyces CX16W	10^6	1.304	0.934	1.822	0.119
		10^8	1.007	0.717	0.415	0.965
	Streptomyces FT05W	10^6	1.237	0.887	1.723	0.209
		10^8	1.011	0.716	1.426	0.952
	Streptomyces LAU18R	10^6	1.193	0.851	1.673	0.305
		10^8	1.260	0.900	1.754	0.178
	Streptomyces SW06W	10^6	1.410	1.013	1.963	0.041
		10^8	1.220	0.873	1.706	0.244
	Streptomyces SW29W	10^6	0.777	0.551	1.094	0.148
		10^8	0.751	0.531	1.062	0.106
	Streptomyces YRU27D	10^6	1.730	1.242	2.409	0.001
		10^8	1.370	0.979	1.915	0.066
	Streptomyces ZEA17I	10^6	0.870	0.613	1.235	0.437
		10^8	0.986	0.703	1.384	0.936
	Actinovate	10^6	1.207	0.863	1.688	0.272
		10^8	1.293	0.924	1.810	0.134

[1] spore concentration

Table 4.3.3 – Disease incidence on lettuce "Batavia"in growth chamber in the experiment B.

			Disease incidence (%)								
		Days[1]	*4*	*7*	*8*	*9*	*10*	*11*	*14*	*16*	*25*
Trials	Inoculated control		25.64	35.90	37.18	37.18	39.74	38.46	47.44	50.00	75.64
	Contans		12.82	12.82	12.82	14.10	14.10	14.10	17.95	21.79	21.79
		(CFU/m^2)[2]									
	Streptomyces	10^6	32.05	34.62	37.18	39.74	42.31	42.31	44.87	48.72	52.56
	ALP07R	10^8	14.10	19.23	19.23	21.79	26.92	26.92	32.05	39.74	39.74
	Streptomyces	10^6	6.41	16.67	17.95	17.95	20.51	24.36	26.92	33.33	53.85
	CVM02R	10^8	20.51	23.08	25.64	24.36	28.21	30.77	33.33	37.18	48.72
	Streptomyces	10^6	20.51	25.64	30.77	30.77	34.62	37.18	42.31	46.15	53.85
	CX14W	10^8	17.95	29.49	32.05	33.33	37.18	37.18	42.31	48.72	50.00
	Streptomyces	10^6	37.18	38.46	38.46	39.74	39.74	46.15	46.15	48.72	51.28
	CX16W	10^8	29.49	30.77	44.87	47.44	50.00	50.00	50.00	57.69	66.67
	Streptomyces	10^6	16.67	19.23	20.51	23.08	25.64	26.92	35.90	44.87	44.87
	FT05W	10^8	15.38	16.67	21.79	21.79	25.64	28.21	32.05	33.33	44.87
	Streptomyces	10^6	24.36	28.21	28.21	29.49	33.33	33.33	37.18	37.18	39.74
	LAU18R	10^8	19.23	19.23	21.79	24.36	26.92	29.49	39.74	46.15	74.36
	Streptomyces	10^6	24.36	24.36	30.77	30.77	34.62	34.62	34.62	34.62	34.62
	SW06W	10^8	19.23	24.36	25.64	25.64	28.21	29.49	41.03	48.72	71.79
	Streptomyces	10^6	15.38	19.23	17.95	19.23	21.79	23.08	32.05	34.62	35.90
	SW29W	10^8	23.08	28.21	33.33	37.18	37.18	37.18	38.46	47.44	65.38
	Streptomyces	10^6	26.92	29.49	38.46	39.74	39.74	39.74	46.15	46.15	58.97
	YRU27D	10^8	33.33	35.90	35.90	38.46	38.46	41.03	46.15	50.00	61.54
	Streptomyces	10^6	24.36	32.05	34.62	34.62	33.33	33.33	37.18	42.31	53.85
	ZEA17I	10^8	12.82	17.95	19.23	20.51	24.36	24.36	30.77	34.62	53.85
	Actinovate	10^6	44.87	44.87	44.87	43.59	46.15	47.44	47.44	47.44	62.82
		10^8	16.67	19.23	21.79	21.79	24.36	25.64	25.64	30.77	39.74

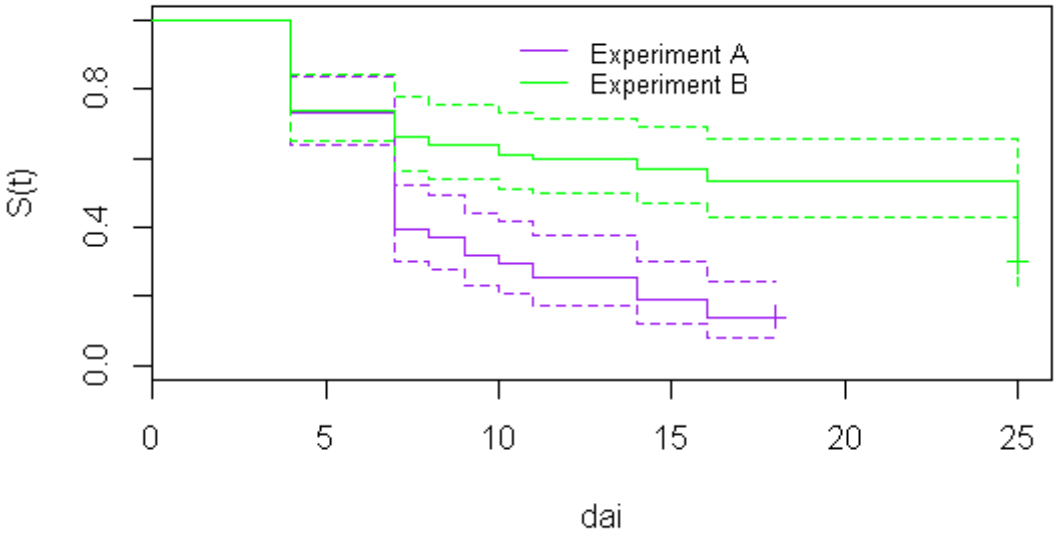
[1] days after sowing [2] spore concentration

Table 4.3.4 – The hazard ratio and confidence intervals from the Cox model for lettuce "Batavia" in growth chamber in the experiment B.

			Hazard Ratio (HR)	**Lower 0.95**	**Upper 0.95**	**P**
Trials	Contans		0.343	0.211	0.554	1.28e-0.5
		(CFU/m^2)[1]				
	Streptomyces ALP07R	10^6	0.941	0.636	1.391	0.760
		10^8	0.458	0.293	0.716	0.00062
	Streptomyces CVM02R	10^6	0.508	0.333	0.775	0.0017
		10^8	0.813	0.548	1.208	0.306
	Streptomyces CX14W	10^6	1.064	0.733	1.543	0.745
		10^8	0.650	0.428	0.988	0.044
	Streptomyces CX16W	10^6	0.731	0.484	1.103	0.136
		10^8	1.279	0.886	1.848	0.189
	Streptomyces FT05W	10^6	0.598	0.393	0.908	0.0159
		10^8	0.420	0.266	0.663	0.00019
	Streptomyces LAU18R	10^6	0.555	0.361	0.852	0.0072
		10^8	0.689	0.462	1.028	0.068
	Streptomyces SW06W	10^6	0.402	0.251	0.641	0.00013
		10^8	0.926	0.632	1.355	0.691
	Streptomyces SW29W	10^6	0.411	0.259	0.653	0.0002
		10^8	0.824	0.562	1.208	0.321
	Streptomyces YRU27D	10^6	0.579	0.380	0.883	0.011
		10^8	1.175	0.816	1.693	0.386
	Streptomyces ZEA17I	10^6	0.836	0.567	1.233	0.366
		10^8	0.720	0.485	1.069	0.103
	Actinovate	10^6	0.926	0.625	1.373	0.704
		10^8	0.404	0.256	0.638	9.93e-05

[1] spore concentration

Figure 4.3.1 – Kaplan-Meier survival curves on lettuce “Batavia” in inoculated controls of experiment A and experiment B.(dai = days after pathogen inoculation, S(t) = survivor function)



4.4 Field experiment

4.4.1 Materials and methods

Plant material and growth conditions

Seedlings were grown in polystyrene seed tray (84 cells). Each cell (48cm^3) was filled with Irish and Baltic peats based growing substrate (Vigorplant, Italy). After antagonist inoculation and sowing, the surface of every cell was first covered with a thin layer of growing substrate and then with coarse perlite. The seed tray was incubated in growth chamber (24°C, 55% relative humidity and 15 h photoperiod) until transplanting and watered every 2-3 days with tap water.

Antagonist inoculation

Streptomycete spore suspension was applied by three different methodologies.

A. The seed tray was watered with tap water to wet the growing substrate. Subsequently, each cell was inoculated with 0.5 mL of spore suspension (10^4 CFU), uniformly distributed on the top of growing medium.
B. Seeds were surface-sterilized in 0.7% sodium hypochlorite for 5 minutes and then rinsed 3 times in sterile water. In a Petri dish, fifty seeds were inoculated with 1 mL of spore suspension (10^7CFU/mL). Seeds were left to dry under the laminar flow hood. Control seeds were treated with sterile water.
C. The seed tray (32x53x5.5) cm was dipped in 500 mLcontaininig 10^8 CFU spore suspension.

Actinovate (Natural Industries, Inc. USA) based on *Streptomyces lydicus* WYEC 108 was used as reference commercial product. It was inoculated on the top of growing medium (5Kg/ha) or as spore suspension (see Paragraph 1.3 for details) for treatment B.

Pathogen inoculum preparation and seedling infection

The *S. sclerotiorum* inoculum was prepared as previously described. One hundred mg of slurry, dissolved in a suitable volume of sterile water to facilitate the distribution, was uniformly applied on the top of the growing substrate surface of every cell.

Transplanting in field

Experiments were carried out in a field located in Travacò Siccomario, Pavia (Italy), on a loamy soil, with neutral pH. Plant density was 5.55 plants/m^2. One drip line (emitter spacing 0.2 m) was put close to the plants in row to uniformly distribute the water and to maintain field water capacity in the soil.

Disease assessment and weight measurement

After pathogen inoculation, plants showing typical symptoms of basal drop were recorded periodically and disease incidence was calculated. At the end of the experiment, plants were harvested and the head weight was determined and compared to the un-inoculated control.

Statistical analysis

The dynamics of disease was analyzed by survival analysis as previously described. The weights data were submitted to ANOVA, followed by Tukey post-hoc test for multiple comparison (P = 0.05), using the TukeyC package (Faria *et al.*, 2013). The heads weights in treated trials were compared to the un-inoculated one.

4.4.2 Experiment 1

Aim of the work: to study the activity of *Streptomyces* strains against lettuce drop.

Description of the experiment

Lettuce "Butterhead" plants (*Lactuca sativa* var. *capitata* L.) (Franchi sementi, Italy) were sown on 4 September 2013 and incubated in growth chamber for 23 days. Streptomycete strains, as well as the commercial product Actinovate, were applied according to the mode of inoculation A at sowing and 19 days later. Twenty-two days after sowing, each seedling was inoculated with the pathogen. Plants were transplanted on 27 September 2013. In field, 2 plastic tunnels (1.2x12 m) were prepared and trials were organized in a completely randomized blocks design. Plants were harvested on 17 February 2014.

Seven trials were prepared, each consisted of 20 plants.

1. Un-inoculated control - plants not inoculated
2. Inoculated control - plants inoculated with *S. sclerotiorum*
3. *Streptomyces* ALP07R - plants inoculated with *S. sclerotiorum* and *Streptomyces* ALP07R
4. *Streptomyces* FT05W - plants inoculated with *S. sclerotiorum* and *Streptomyces* FT05W
5. *Streptomyces* LAU18R - plants inoculated with *S. sclerotiorum* and *Streptomyces* LAU18R
6. *Streptomyces* ZEA17I - plants inoculated with *S. sclerotiorum* and *Streptomyces* ZEA17I
7. Actinovate - plants inoculated with *S. sclerotiorum* and Actinovate

Results

The first symptoms of lettuce drop appeared 10 days after *Sclerotinia sclerotiorum* inoculation (dai), and were observed in all trials. The lettuce drop incidence in the inoculated control was 15% 10 dai and reached 60% at the end of the experiment (Table 4.4.1). Significant differences were

observed for dynamics of disease among the trials (χ^2 = 23.7, P = 0.0002) (Figure 4.4.1). The application of some antagonists caused higher lettuce drop incidence, whereas for others, the disease incidence was higher only in first weeks after pathogen inoculation (Table 4.4.1) Actinovate and *Streptomyces* LAU18R significantly increased the risk of lettuce drop (P = 0.04 and P = 0.01, respectively) (Table 4.4.2): the values of HR were 2.10 for the commercial product and 2.61 for the antagonist, meaning that their application more than doubled disease incidence. Similarly, *Streptomyces* ALP07R increased lettuce drop incidence by 52%, but it was not significantly different from the control (HR = 1.52, P = 0.28) (Table 4.4.2). *Streptomyces* ZEA17I showed higher disease incidence than the inoculated control until 56 dai, thereafter the disease incidence was lower than the inoculated control (Table 4.4.1). However, the HR value was not significant (HR = 0.93, P = 0.86). The only trial in which the disease incidence was always, but not significantly, lower than the inoculated control was *Streptomyces* FT05W (HR = 0.44, P = 0.1) (Table 4.4.2). The application of this antagonist reduced the risk of lettuce drop by 56% (Table 4.4.2).

At harvest, mean weight of lettuce heads for the un-inoculated control was 413.17 g, whereas those treated with *Streptomyces* ALP07R, *Streptomyces* FT05W, *Streptomyces* ZEA17I and Actinovate were higher; 425.60 g, 462.15 g, 459.40 g and 521.00 g, respectively and those treated with *Streptomyces* LAU18R were lighter, 281.00 g (Table 4.4.3). Only the data of trials with similar number of harvested plants were submitted to ANOVA: un-inoculated control, *Streptomyces* FT05W and *Streptomyces* ZEA17I (12, 13 and 10 plants, respectively) but the differences among weights were not significant (P = 0.667). Concerning the roots mean weight, it was 18.50 g in the un-inoculated control whereas in the trials treated with the antagonists it was lower (Table 4.4.3). No significant differences resulted among the un-inoculated control, *Streptomyces* FT05W and *Streptomyces* ZEA17I (P = 0.1).

Table 4.4.1 – Disease incidence on lettuce "Butterhead". Travacò Siccomario (Pv), October 2013-February 2014.

		Disease incidence (%)								
		10 dai	16 dai	20 dai	30 dai	45 dai	56 dai	73 dai	122 dai	142 dai
Trials	Inoculated control	15	20	25	25	40	40	55	60	60
	Streptomyces ALP07R	30	40	40	50	65	70	70	70	70
	Streptomyces FT05W	15	25	25	25	25	30	30	30	30
	Streptomyces LAU18R	30	40	40	65	80	80	95	95	95
	Streptomyces ZEA17I	35	45	45	45	45	45	45	50	50
	Actinovate	35	40	40	50	60	60	65	85	95

dai= days after pathogen inoculation

Table 4.4.2 – The hazard ratio and confidence intervals from the Cox model for lettuce "Butterhead". Travacò Siccomario (Pv), October 2013-February 2014.

		Hazard Ratio (HR)	**Lower 0.95**	**Upper 0.95**	**P**
Trials	*Streptomyces* ALP07R	1.52	0.70	3.30	0.28
	Streptomyces FT05W	0.44	0.16	1.17	0.10
	Streptomyces LAU18R	2.61	1.26	5.43	0.01
	Streptomyces ZEA17I	0.93	0.40	2.15	0.85
	Actinovate	2.10	1.02	4.33	0.044

Figure 4.4.1 – Kaplan- Meier survival curves on lettuce "Butterhead". Travacò Siccomario (Pv), September 2013 - February 2014 (dai = days after pathogen inoculation, S(t) = survivor function).

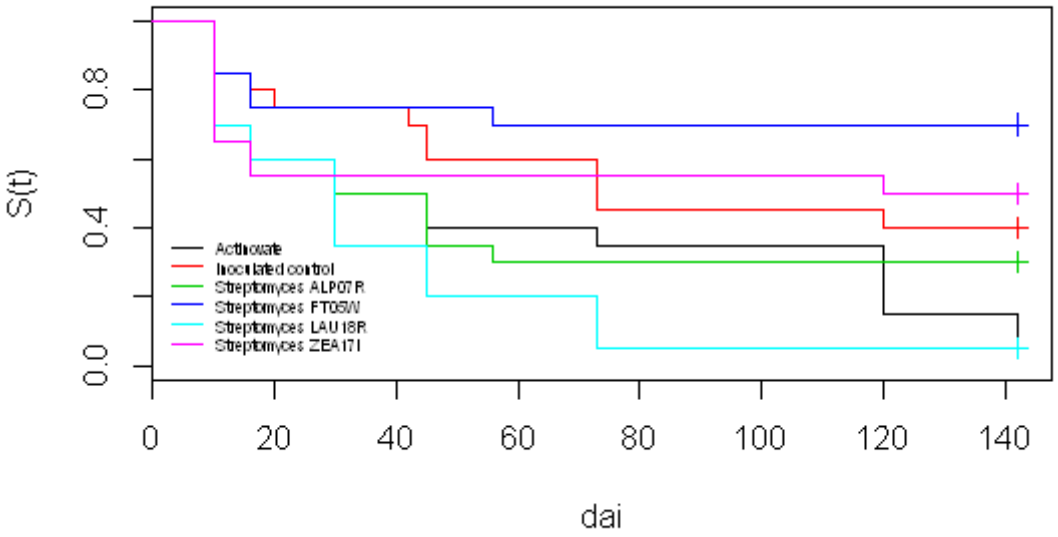


Table 4.4.3 – Mean weight of lettuce "Butterhead" heads. Travacò Siccomario (Pv). 17 February 2014.

		N[1]	Weight (g)			
			Head	Standard error	Roots	Standard error
Trials	Un-inoculated control	12	413.17 ns[2]	52.31	18.50 ns	0.75
	Streptomyces ALP07R	5	425.60	9.49	16.00	1.76
	Streptomyces FT05W	13	462.15 ns	34.22	16.38 ns	0.83
	Streptomyces LAU18R	1	281.00	/	12.00	/
	Streptomyces ZEA17I	10	459.40 ns	42.34	16.00 ns	1.17
	Actinovate	1	521.00	/	17.00	/

[1]= number of harvested plants. [2] = ANOVA was not significant (P>0.05).

4.4.3 Experiment 2

Aim of the experiment: to study the effect of plant-antagonist interaction timing on disease control

Description of the experiment

Lettuce "Ice queen" plants (*Lactuca sativa* var. *capitata* L.) (Franchi sementi, Italy) were sown on 19 February 2014 and grown in growth chamber for 29 days. Streptomycete strains, as well as the commercial product Actinovate, were applied according to the mode of inoculation A at sowing and 14 days after. According to the time of pathogen inoculation, two experiments were performed. In the Experiment A *Sclerotinia sclerotiorum* was inoculated 28 days after sowing, whereas in the Experiment B it was inoculated 37 days after sowing. Plants were transplanted on 20 March 2014. For each experiment, two plastic tunnels (1.2x12 m) were prepared and trials were organized in a completely randomized blocks design. The plastic tunnels were removed on 6 May 2014 due to high temperatures. Plants of the experiment A were harvested on 17 May 2014 whereas for the experiment B on 24 May 2014.

For both experiments, eight trials were prepared, each composed of 20 plants.

1. Un inoculated control - plants not inoculated
2. Inoculated control - plants inoculated with *S. sclerotiorum*
3. *Streptomyces* CVM02R - plants inoculated with *S. sclerotiorum* and *Streptomyces* CVM02R
4. *Streptomyces* FT05W - plants inoculated with *S. sclerotiorum* and *Streptomyces* FT05W
5. *Streptomyces* SW06W - plants inoculated with *S. sclerotiorum* and *Streptomyces* SW06W
6. *Streptomyces* SW29W - plants inoculated with *S. sclerotiorum* and *Streptomyces* SW29W
7. *Streptomyces* ZEA17I - plants inoculated with *S. sclerotiorum* and *Streptomyces* ZEA17I
8. Actinovate - plants inoculated with *S. sclerotiorum* and Actinovate

Results

In the experiment A, when *Sclerotinia sclerotiorum* was inoculated 14 days after the second antagonists application, typical symptoms of lettuce drop were observed 8 days later (dai) (Table 4.4.5). At this time, the disease incidence was similar and even 5-25% higher in trials inoculated with the antagonists than the inoculated control. During the next week, the same situation was observed only for *Streptomyces* FT05W, *Streptomyces* SW06W and *Streptomyces* ZEA17I which showed values 5-10% higher than the inoculated control. In the other trials disease incidence was 5-10% lower than the inoculated control. This situation persisted during the next weeks until 23 dai in some trials. Only for *Streptomyces* CVM02R disease incidence was lower than the control during

the entire experiment (Table 4.4.4). In the inoculated control the lettuce drop incidence reached 75% 30 dai and remained stable for the next 28 days, until the end of the experiment. At the end of the experiment disease incidence for *Streptomyces* FT05W was 80% and it was the only trial with incidence higher than the control. On the contrary, the other trials showed 5-18% lower incidence than the control. For every trial, the KM survival curves were constructed from the data of dead plants (Figure 4.4.2), but no significant differences were found among them ($\chi^2 = 3.6$, P = 0.734).

At harvest, the mean weight of lettuce heads for the trials *Streptomyces* CVM02R, *Streptomyces* SW29W and *Streptomyces* ZEA17I was higher than the un-inoculated control, and were 419.25 g, 442.28 g and 458.57 g, respectively, compared to 403.87 g of the control (Table 4.4.5). On the contrary, the mean weights of *Streptomyces* FT05W, *Streptomyces* SW06W and Actinovate were lower 371.00 g, 392.83 g and 359.00 g, respectively (Table 4.4.5), although these values were not significantly different (P = 0.967) from the others.

Table 4.4.4 – Disease incidence on lettuce" Ice queen" in the experiment A. 20 March 2014 -17 May 2014, Travacò Siccomario (Pv).

		Disease incidence (%)								
		8 dai	13 dai	16 dai	23 dai	30 dai	37 dai	44 dai	51 dai	58 dai
Trials	Inoculated control	5	40	45	45	75	75	75	75	75
	Streptomyces CVM02R	15.79	31.58	36.84	42.11	57.89	57.89	57.89	57.89	57.89
	Streptomyces FT05W	15	50	55	65	75	80	80	80	80
	Streptomyces SW06W	30	50	55	55	70	70	70	70	70
	Streptomyces SW29W	15	35	45	50	55	60	65	65	65
	Streptomyces ZEA17I	10	45	55	55	60	60	60	65	65
	Actinovate	5	35	40	45	70	70	70	70	70

dai= days after pathogen inoculation

Figure 4.4.2 – Kaplan-Meirer survival curves in the experiment A. (dai = days after pathogen inoculation, S(t) = survivor function)

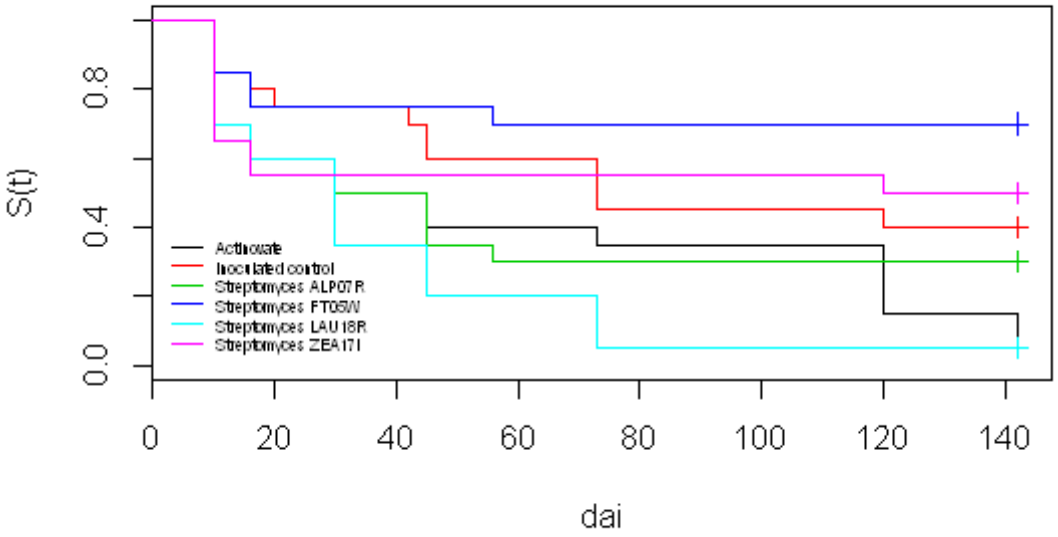


Table 4.4.5 – Mean weight of lettuce" Ice queen" heads in the experiment A. Travacò Siccomario (Pv), 17 May, 2014.

	Trials						
	Un-inoculated control	*Streptomyces* CVM02R	*Streptomyces* FT05W	*Streptomyces* SW06W	*Streptomyces* SW29W	*Streptomyces* ZEA17I	Actinovate
N[1]	15	8	4	6	7	7	6
Weight (g)	403.87 ns[2]	419.25 ns	371.00 ns	392.83 ns	442.28 ns	458.57 ns	359.00 ns
Standard error	58.61	81.85	91.96	61.93	45.23	61.90	57.60

[1]= number of harvested plants. [2] = ANOVA was not significant (P>0.05).

In the experiment B, when *S. sclerotiorum* was inoculated 23 days after the second application of the antagonists, disease incidence was generally lower than in the experiment A (Table 4.4.6). Eight dai, dead plants were observed in the inoculated control and in *Streptomyces* FT05W, *Streptomyces* SW06W and *Streptomyces* SW29W, all with 5% incidence. In the other trials the first symptoms were observed 22 dai. Generally, in all trials treated with the antagonists, the disease incidence was similar or 10-20% lower than the inoculated control and this dynamics was observed until the end

of the experiment. Only *Streptomyces* FT05W showed disease incidence 10-15% higher than the inoculated control from 43 dai until the end of the experiment.
The comparison of survival curves among the inoculated control and the trials inoculated with the antagonists did not show significant differences ($\chi^2 = 4$, P = 0.67) (Figure 4.4.3).

At harvest, the mean weight of lettuce heads of the un-inoculated control was 404.13 g (Table 4.4.7). For the plants treated with the antagonists the values were higher: 440.28 g for *Streptomyces* CVM02R, 495.92 g for *Streptomyces* SW06W, 456.64 g for SW29W, 409.17 g for *Streptomyces* ZEA17I and 429.58 for Actinovate. Only the mean weight of head treated with *Streptomyces* FT05W was lower than the un-inoculated control, 390.33 g, although it was not significantly different from the control (P = 0.963).

Table 4.4.6 – Disease incidence on lettuce" Ice queen" in the experiment B. 27 March 2014-24 May 2014, Travacò Siccomario (Pv).

		Disease incidence (%)							
		8 dai	15 dai	22 dai	29 dai	36 dai	43 dai	50 dai	57 dai
Trials	Inoculated control	5	5	35	35	35	35	40	40
	Streptomyces CVM02R	0	0	15	15	20	20	30	30
	Streptomyces FT05W	5	5	15	20	20	45	50	55
	Streptomyces SW06W	5	5	20	35	35	35	35	35
	Streptomyces SW29W	5	5	15	20	25	30	30	30
	Streptomyces ZEA17I	0	0	15	15	15	20	30	40
	Actinovate	0	0	15	15	15	20	30	40

dai= days after pathogen inoculation

Figure 4.4.3 – Kaplan-Meirer survival curves in the experiment B. (dai = days after pathogen inoculation, S(t) = survivor function)

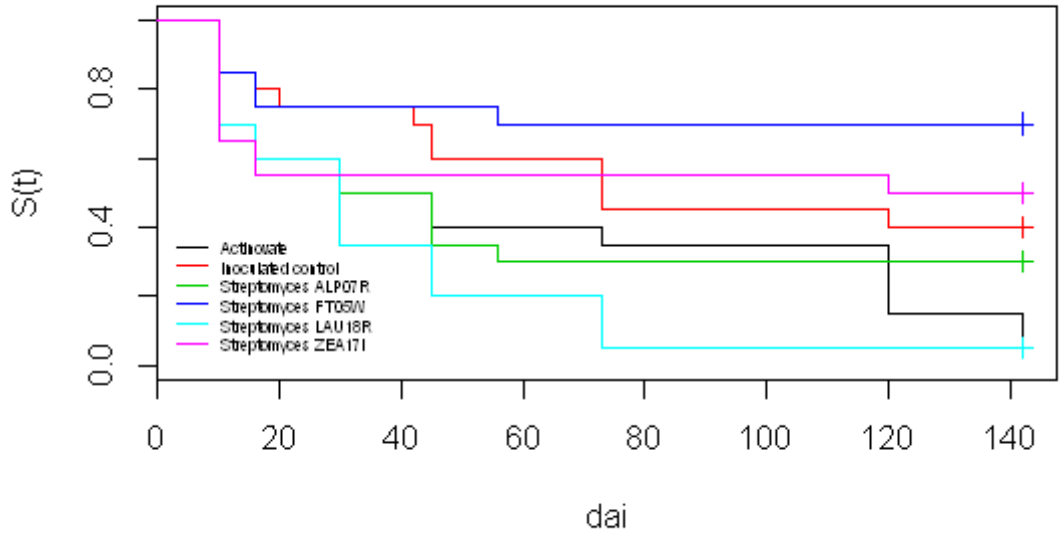


Table 4.4.7 – Mean weight of lettuce" Ice queen" in the experiment B. Travacò Siccomario (Pv) 24 May, 2014

	Trials						
	Un-inoculated control	*Streptomyces* CVM02R	*Streptomyces* FT05W	*Streptomyces* SW06W	*Streptomyces* SW29W	*Streptomyces* ZEA17I	Actinovate
N[1]	15	14	9	13	14	12	12
Weight (g)	404.13 ns[2]	440.28 ns	390.33 ns	495.92 ns	456.64 ns	409.17 ns	429.58 ns
Standard error	50.66	72.48	82.22	78.06	63.75	70.06	95.21

[1]= number of harvested plants. [2] = ANOVA was not significant (P>0.05).

4.4.4 Experiment 3

Aim of the work: to study the effect of plant-antagonist interaction timing on disease control

Description of the experiment

Lettuce" Ice queen" plants (*Lactuca sativa* var. *capitata* L.) (Franchi sementi, Italy). Streptomycete strains, as well as the commercial product Actinovate, were applied according to the mode of inoculation B at sowing and 20 days after, according to the mode C.

Regarding the time of pathogen inoculation, two experiments were performed. In the Experiment A, plants were grown for 28 days in growth chamber and inoculated with the pathogen 27 days after sowing. In the experiment B, plants were grown for 21 days and inoculated 28 days after sowing. For each experiment, trials were organized in a completely randomized blocks design.

For both experiments, eight trials were prepared, each composed of 20 plants.

9. Un-inoculated control - plants not inoculated
10. Inoculated control - plants inoculated with *S. sclerotiorum*
11. *Streptomyces* CVM02R - plants inoculated with *S. sclerotiorum* and *Streptomyces* CVM02R
12. *Streptomyces* FT05W - plants inoculated with *S. sclerotiorum* and *Streptomyces* FT05W
13. *Streptomyces* SW06W - plants inoculated with *S. sclerotiorum* and *Streptomyces* SW06W
14. *Streptomyces* SW29W - plants inoculated with *S. sclerotiorum* and *Streptomyces* SW29W
15. *Streptomyces* ZEA17I - plants inoculated with *S. sclerotiorum* and *Streptomyces* ZEA17I
16. Actinovate - plants inoculated with *S. sclerotiorum* and Actinovate

Results

During entire crop cycle, no symptoms of lettuce drop were observed either in experiment A or in experiment B. At harvest time, only lettuce heads showing no pre-flowering signs were weighted. The mean weight of lettuce heads was 562.33 g for the un-inoculated control, while it was lower for the other trials values: 562.00 g for *Streptomyces* CVM02R, 534.31 g for *Streptomyces* FT05W, 511.88 g for *Streptomyces* SW06W, 487.50 g for *Streptomyces* SW29W, 521.80 g for *Streptomyces* ZEA17I and 437.00 g for Actinovate (Table 4.4.8). Mean values were not significantly different among them (P = 0.326). The mean weight of lettuce heads in the un-inoculated control of the experiment B was 374.13 g and all the trial showed higher, but not significantly different, values. *Streptomyces* SW06W increased the heads weight on average by 134 g (Table 4.4.9).

Table 4.4.8 – Mean weight of lettuce" Ice queen" in the experiment B. Travacò Siccomario (Pv), 1 August 2014.

	Trials						
	Un-inoculated control	*Streptomyces* CVM02R	*Streptomyces* FT05W	*Streptomyces* SW06W	*Streptomyces* SW29W	*Streptomyces* ZEA17I	Actinovate
N[1]	15	11	16	17	12	10	9
Weight (g)	562.33 ns[2]	562.00 ns	534.31 ns	511.88 ns	487.50 ns	521.80 ns	437.00 ns
Standard error	30.55	43.31	25.54	33.22	45.43	28.98	60.75

[1]= number of harvested plants. [2] = ANOVA was not significant (P>0.05).

Table 4.4.9 – Mean weight of lettuce "Ice queen" in the experiment A. Travacò Siccomario (Pv), 1 August 2014.

	Trials						
	Un-inoculated control	*Streptomyces* CVM02R	*Streptomyces* FT05W	*Streptomyces* SW06W	*Streptomyces* SW29W	*Streptomyces* ZEA17I	Actinovate
N[1]	8	6	7	9	5	6	5
Weight (g)	374.13 ns[2]	493.50 ns	408.00 ns	508.56 ns	486.00 ns	377.67 ns	393.00 ns
Standard error	61.80	59.91	51.64	56.53	126.81	24.14	108.86

[1]= number of harvested plants. [2] = ANOVA was not significant (P>0.05).

4.5 Conclusions

In these experiments the activity of a pool of *Streptomyces* strains was studied *in vivo*, which showed 75% to 90% antagonistic inhibition of *Sclerotinia sclerotiorum* in dual culture assays. We observed a lack of correlation between the *in vitro* and *in vivo* trials. In fact, *Streptomyces* LAU18R, the most active strain with 90% inhibition *in vitro*, *in vivo* favored a substantial increase of lettuce drop in field. The same negative effect was observed for *Streptomyces* SW06W and *Streptomyces*

YRU27D, both with 90% and 87.50% of activity *in vitro*, respectively, in growth chamber experiment. In contrast, *Streptomyces* FT05W, the less active in dual culture assay, resulted one of the most active strains against lettuce drop in growth chamber experiment when applied one week before sowing in infected substrate.

The growth chamber experiments showed that, when the plants were sown one week after substrate inoculation with antagonist and *S. sclerotiorum*, it generally resulted in a better protection against lettuce drop. On the contrary, when pathogen and antagonists were applied on the day of sowing, lettuce drop incidence was higher than the inoculated control. It can be hypothesized that during the early root tissue and rhizosphere colonization, the antagonists or their interaction with the pathogen might temporarily negatively affect the host response to pathogen, possibly through the production of temporary toxic metabolites (Kremer and Souissi, 2001; Schippers *et al.*, 1987). These results may also suggests, that the antagonists may need time to grow, colonize the soil and release bioactive compounds involved in biological control. However, it is important to highlight that the disease incidence in the inoculated control between the two experiments were significantly different, resulting in better protection by the antagonists when the disease pressure was lower.

Based on the growth chamber experiment results, when lettuce was sown one week after substrate inoculation with both the pathogen and the antagonist, we selected the most active strains to study their activity in field. Here, we tried to extend the time of interaction between antagonist and host plant before *S. sclerotiorum* inoculation on the basis of the previous hypothesis. When the pathogen was inoculated few days after the second application of the antagonist, it resulted in significantly higher risk of disease, especially for *Streptomyces* LAU18R and Actinovate, or did not significantly improve plant protection. Unfortunately, even when the pathogen was inoculated three weeks after the second treatment with the antagonists, no significant differences were observed for lettuce drop dynamics. Finally, in these experiments non plant growth promotion was observed for these antagonists, but some strains such as Actinovate, *Streptomyces* LAU18R and *Streptomyces* FT05W negatively affect head weight, even though not significantly.

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5 Chapter 5: Spatio-temporal colonization of lettuce rhizosphere and roots by *Streptomyces* sp. ZEA17I

5.1 Introduction

Despite the optimal performance at laboratory-scale screening tests, beneficial bacteria such as Biological Control Agents (BCAs) and Plant Growth-Promoting Bacteria (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 presence of competing microorganisms or lower rhizosphere competence, that is poor colonization of root tissues and rhizosphere of the host plant (Compant *et al.*, 2010; Ghirardi *et al.*, 2012; Lugtenberg *et al.*, 2001). To overcome these obstacles, it is essential to understand how the bacterium interacts 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 traits were used (Ghirardi *et al.*, 2012). In recent years, several traits essential for rhizosphere colonization were identified in *Pseudomonas* spp. (Lugtenberg and Kamilova, 2009), however similar studies are missing for other genera of bacteria.

Several markers have been developed and adopted to study localization and quantification of beneficial bacteria in the rhizosphere; among these, antibiotic resistance has been widely used (Gamalero *et al.*, 2003; Prosser, 1994). However, the natural resistance among rhizosphere microorganisms may prevent its use. Currently, fluorescent markers are gaining increasing popularity for colonization studies (Cao *et al.*, 2011; Krzyzanowska *et al.*, 2012; Lu *et al.*, 2004). They are based on the green fluorescent protein (GFP), which converts blue chemiluminescence of the Ca^{2+}-sensitive photoprotein aequorin into green light (Chalfie *et al.*, 1994). Several studies utilized GFP to study bacterial colonization of roots and rhizosphere in sterile conditions (Coombs and Franco, 2003b; Weyens *et al.*, 2012). These studies provide basic understanding of interactions between bacteria and the host plant, but they exclude the complex interactions *in vivo*. In non-sterile conditions with high microbial diversity, the bacteria have to compete with other microorganisms present in rhizosphere, and in some cases the competition reduced colonization ability of beneficial bacteria (Cao *et al.*, 2011; Hohmann *et al.*, 2012; Weyens *et al.*, 2012).

The objective of this work was to study the spatio-temporal colonization of lettuce rhizosphere and roots by a *Streptomyces* strain which showed strong inhibition *in vitro* against *Sclerotinia sclerotiorum* (see Chapter 2 details) and beneficial effects on plant growth and health *in vivo* (see Chapter 4 for details). Moreover, the effects of two inoculation methods on the ability of the strain to differentially colonize rhizosphere and roots were compared. To get insight into the localization

and colonization of the strain in non-sterile growing substrate, the strain was transformed with the enhanced green fluorescent protein (EGFP), a GFP derivative adapted engineered for *Streptomyces* spp. (Sun *et al.*, 1999).

5.2 Methodology

5.2.1 Streptomyces culture

Streptomyces sp. ZEA17I was used in the experiment. It was transformed with the pIJ8641 construct containing EGFP gene under a constitutive promoter (Sun *et al.*, 1999) and the fitness of the transformed strain was compared to the wild type (Chen *et al.*, 2014). EGFP-ZEA17I was grown at 24°C on CZY medium with 50 mg apramycin added. Apramycin is the selective marker used to transform *Streptomyces* spp. (Sun *et al.*, 1999). After ten days growth in a Petri plate, aerial mycelium and spores were collected in 10% sterile glycerol and filtered through 2 layers of gauze. The concentration was determined and spore suspension was stored at -20°C.

5.2.2 Assessment of apramycin resistant streptomycetes in growing substrate

The non-sterilized Irish and Baltic peats based growing substrate (Vigorplant, Italy) was used. To verify that apramycin resistant streptomycetes were not naturally present, a portion of substrate was collected, resuspended in sterile water and plated on WA medium with 25 mg nalidixic adic, 50 g apramycin, 50 mg nystatin and 50 mg cycloheximide added. Plates were incubated for 7 days at 24°C and the presence of streptomycete colonies were visually checked.

5.2.3 Bulk soil colonization

The growth substrate was placed in a polystyrene seed tray (48cm^3/cell) and watered with tap water. In every cell, EGFP-ZEA17I was uniformly distributed on the top of the substrate adding 1 mL spore suspension (1x10^7 CFU/mL). The growth substrate was incubated in growth chamber (24°C, 55% relative humidity and 15 h photoperiod) and watered every 2-3 days with tap water. EGFP-ZEA17I was re-isolated 4 hours (day 0), 10, 20, and 30 days post inoculation (dpi) 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 weight/volume) 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 EGFP-ZEA17I concentration was expressed as CFU/g of growth substrate dry weight (DW).

5.2.4 Plant inoculation

Lettuce "Ice queen" 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 minutes and rinsed three times in sterile water. Two methods were used to inoculate the EGFP-ZEA17I strain. In the growth substrate inoculation method, 1 mL of spore suspension ($1x10^7$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 EGFP-ZEA17I spore suspension ($1x10^7$ CFU/mL) for 5 min. and then left to dry under the laminar flow hood. After sowing one seed per cell of the tray, the seedlings were incubated and watered as described previously.

To determine the inoculum load, EGFP-ZEA17I was re-isolated from seeds and growth substrate after the inoculum application. In growth substrate inoculation method, the EGFP-ZEA17I strain was re-isolated four hours after the soil inoculation as described above for bulk soil, and its amount was expresses as CFU/g of the growth substrate. In 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 EGFP-ZEA17I colonies were counted and the amount was expressed first as CFU/seed and then recalculated as CFU/g of growth substrate DW .

5.2.5 EGFP-ZEA17I re-isolation from rhizosphere and root tissues.

The EGFP-ZEA17I strain was re-isolated 10, 20, and 30 days after sowing from rhizosphere and root tissues of 6 lettuce seedlings, equal to number of replicates. Seedling and its root system were carefully taken off the cell and the bulk soil was removed by gently shaking the plantlet (Bulgarelli *et al.*, 2012). For rhizosphere, each seedling was cut at neck level and 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). Roots were removed and the suspension was filtered through a 300 μm nylon mesh to obtain the soil rhizosphere whose dry weight was determined. The suspension was centrifuged at 10000 rpm 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. Plates were incubated at 24°C for 7 days. The EGFP-ZEA17I colonies were counted and the concentration was expressed as CFU/g of rhizosphere DW.

For the inner root tissues, roots were surface sterilized with propylene oxide for one hour (Sardi *et al.*, 1992). Roots 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 EGFP-ZEA17I concentration was determined as described previously and expressed as CFU/g of roots DW.

5.2.6 Statistical analysis

Statistical analysis were done using R software, version R3.0.2. (R_Core_Team, 2013). 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).

5.3 Results

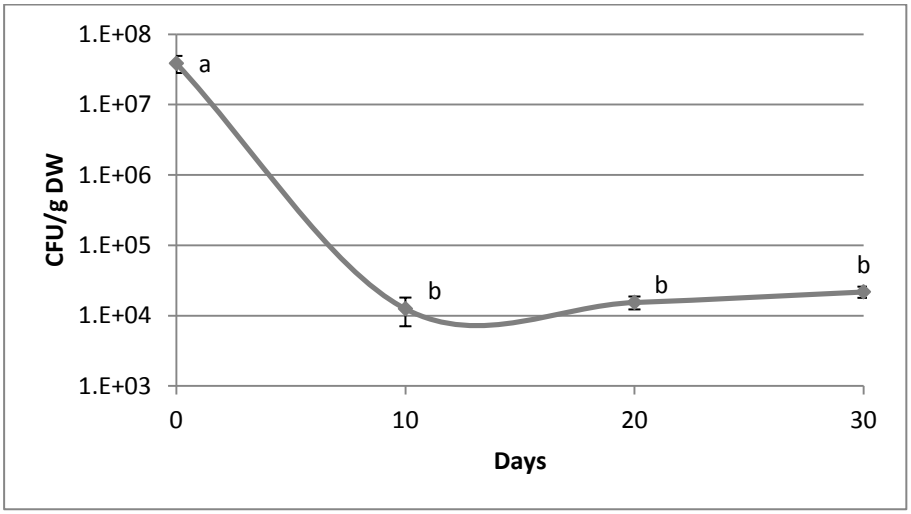
5.3.1 Assessment of apramycin resistant streptomycetes in growing medium

Apramycin is the selective marker used to transform *Streptomyces* spp. (Sun *et al.*, 1999). However, to use apramycin as a selection marker to identify the inoculated EGFP-ZEA17I strain in greenhouse experiments, the presence of naturally occurring apramycin-resistant *Streptomyces* spp. in the growth substrate was checked, but none were recovered. Therefore non-sterilized growth substrate was used in following experiments.

5.3.2 Colonization dynamics of *Streptomyces* sp. EGFP-ZEA17I in bulk soil

The colonization dynamics of *Streptomyces* sp. EGFP-ZEA17I in bulk soil showed that the initial inoculum of 1.16×10^8 CFU/g DW, decreased significantly within the first 10 days and thereafter it remained stable up to 30 days after inoculation at concentration 10^4 CFU/g DW (Figure 5.3.1)

Figure 5.3.1 - Dynamics of *Streptomyces* sp. EGFP-ZEA17I colonization in bulk soil.



Vertical bars represent standard errors (N = 6). Tukey post-hoc test; means with the same letters are not significantly different (P = 0.05).

5.3.3 Colonization of lettuce rhizosphere and inner root tissues by the EGFP-ZEA17I strain

Streptomyces sp. EGFP-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 inner root tissues of lettuce seedlings differed. In rhizosphere, the concentration of the EGFP-ZEA17I strain remained similar to the inoculated amount during the first 20 days after inoculation with either method. When EGFP-ZEA17I was distributed on top of the growth substrate, a significant increase in concentration was observed 30 days after inoculation. 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 (Figure 5.3.1).

Table 5.3.1 – Colonization dynamic of *Lactuca sativa* var. *capitata* rhizosphere by *Streptomyces* sp. EGFP-ZEA17I.

	Rhizosphere (CFU/ g DW)			
	0 dai[1]	10 dai	20 dai	30 dai
Growth substrate inoculation	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] days after inoculation [2] Tukey post-hoc test; means in one line with the same letters are not significantly different (P = 0.05).

Similarly, the dynamics of EGFP-ZEA17I colonization was studied 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 EGFP-ZEA17I strain was re-isolated from inner root tissues of surface-sterilized roots independently of the inoculation method used, confirming its ability to endophytically colonize lettuce roots. The concentration of EGFP-ZEA17I declined steadily through time, however, this reduction was not significant in either inoculation method (Table 5.3.2).

Table 5.3.2 – Colonization dynamics of *Lactuca sativa* var. *capitata* roots by *Streptomyces* sp. EGFP-ZEA17I.

	Roots (CFU/g DW)		
	10 dai[1]	20 dai	30 dai
Growth substrate inoculation	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] days after inoculation [2] ns - ANOVA was not significant ($P > 0.05$)

Finally, we compared the two *Streptomyces* strain inoculation methods to get an insight if one of the methods improves survival and colonization of the strain in rhizosphere and in lettuce roots. In the

rhizosphere, significantly more EGFP-ZEA17I were re-isolated at all sampling times using the growth substrate inoculation rather than after seed coating (P = 0.0037; 0.0389 and 0.0005, respectively for sampling time 10, 20 and 30 dai). Similarly, in roots, significantly higher concentration of EGFP-ZEA17I was obteined using the growth substrate inoculation at 20 and 30 dai (P = 0.0415 and P = 0.0604, respectively). However, in spite of higher amount of the strain present in roots using this inoculation method, not all seedlings were colonized. Indeed, we failed to re-isolate EGFP-ZEA17I from roots of three seedlings (one seedling at 20 dai and two seedlings at 30 dai).

5.4 Conclusions

Plant beneficial bacteria have a great potential in agrobiology as PGPB and BCAs and reports about successful suppression of plant diseases in controlled conditions are increasing. However, application of these microbial agents in field often fails to achieve the expected results due to lack of knowledge about their biology and interactions within the rhizosphere, with the host plant and pathogens. There are increasing attempts to study the complex interactions that take place in rhizosphere (Compant *et al.*, 2010; Gamalero *et al.*, 2003). One of such approaches exploits fluorescent markers, such as GFP, to visualize the bacteria and study their colonization and plant-microbe interactions. The work aimed to study the spatio-temporal dynamics of colonization of lettuce roots and rhizosphere by a *Streptomyces* sp. ZEA17I, to better understand if and how it establishes in the rhizosphere and colonizes plant roots. To get insight into the localization and colonization of the strain in non-sterile growing substrate, the streptomycete was transformed with the enhanced green fluorescent protein (EGFP). A non-sterile growth substrate was intentionally used to simulate competition with natural microflora and evaluate the competitiveness of the inoculated *Streptomyces* strain. *Streptomyces* sp. EGFP-ZEA17I freely survive in soil in the absence of the host plant, although a sharp decrease in EGFP-ZEA17I quantity within first 10 days after inoculation in the bulk soil was observed. Thereafter, the population remained stable up to 30 days, probably establishing an equilibrium with the indigenous microflora as observed previously (Merzaeva and Shirokikh, 2006; Yuan and Crawford, 1995). In the presence of the lettuce plants we did not observe the initial rapid decline in EGFP-ZEA17I concentration in the rhizosphere as observed in bulk soil. It is possible that the inoculated strain was chemoattracted to rhizosphere by the growing seedling, where it established a relationship with the host plant roots. By its reisolation from the rhizosphere and inner tissues of surface-sterilized roots, we demonstrated that *Streptomyces* sp. EGFP-ZEA17I is both rhizospheric and endophytic, although it is not known if different localization provides different potential for biocontrol and plant growth promotion. It has

been hypothesized that endophytic bacteria establish more stable relationship with plants than rhizospheric or epiphytic bacteria (Compant *et al.*, 2010; Malfanova *et al.*, 2011).

We detected EGFP-ZEA17I in the inner root tissues of growing seedling already 10 days after inoculation at elevated concentrations. Indeed, Coombs and Franco (Coombs and Franco, 2003b) 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.

Until now, localization of BCAs in roots and seeds was mainly studied by microscopic tools, which give a fundamental insight about the spatial distribution of the microorganism along and inside the growing root, but do not quantify and study the dynamics of the BCA colonization (Compant *et al.*, 2010; Olivain *et al.*, 2006) Instead, we quantified the EGFP-tagged strain in roots and rhizosphere to understand if it can remain in soil in competitive concentrations in comparison to the indigenous microflora, such that it can exert its beneficial effects. However, further studies are needed to establish, which parts of plant root the strain colonizes and ultimately how it interacts with the plant in presence of the pathogen, *S. sclerotiorum*, to evaluate its biological control activity.

Part o f the results of this work were user for the publication:

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Conclusions

In Italy, leafy vegetables are economically important crops grown all year round under intensive cultivation practices. Soil borne pathogens represent one of the major limiting factors for the cultivation of vegetables, often causing severe yield and economic losses. The phase-out of methyl bromide and the restrictions in the use of other soil fumigants, in accordance with the Integrated Pest/Disease Management principles, made the management of soil borne epidemics a serious problem (Colla *et al.*, 2012; Gullino *et al.*, 2003). The application of Biological Control Agents (BCAs) has been recognized as a valuable component of IPM strategies (Cook, 1993). For this reason, numerous researches have been carried out in recent years to select and characterize bacterial and fungal strains as antagonistic microorganisms of soil borne pathogens (Haas and Defago, 2005; Harman *et al.*, 2004; Weller, 2007). Some of them have been developed as biocontrol products and are marketed worldwide for greenhouse and field crops (Paulitz and Belanger, 2001). However, the practical application of these microorganisms in un-controlled conditions often shows lack of consistency and variable results. This is mainly due to poor knowledge about the biology of BCAs and their interaction with the pathogen and the host-plant in a specific agroecosystem. Identifying new BCA is of fundamental importance for the management of soil borne epidemics in horticulture, but it is also important improving the knowledge about their biology such as mechanisms of action as well as the modes and times of application to obtain the best expressions of antagonistic activity (Alabouvette *et al.*, 2006; Spadaro and Gullino, 2005).

This PhD thesis aimed to study bacteria of the genus *Streptomyces* as antagonistic microorganisms and Plant Growth Promoting Bacteria for their application in management of soil borne fungal epidemics in horticulture.

It is well known that streptomycetes are soil inhabitants, where they not only have an important ecological role in the turn-over of organic material, but can also establish relationships with plant roots to promote host growth and protection against pathogens through the production of bioactive compounds, lytic enzymes, phytohormones and siderophores (Crawford *et al.*, 1993; Doumbou *et al.*, 2001; Hopwood, 2007; Tokala *et al.*, 2002b). Therefore, they are considered valuable candidates for the goal of this project.

The selection of bacterial isolated from appropriate source represents an important step toward the screening of beneficial microorganisms and it must take in consideration target pathogens and their epidemiology (Kohl *et al.*, 2011; Pliego *et al.*, 2011). Since we were searching for microorganisms against soil borne pathogens, which attack plants at root level, we used *Streptomyces* strains showing endophytic habitus in roots (Petrolini *et al.*, 1996; Sardi *et al.*, 1992).

The first step of the work concerned the massive *in vitro* screening of 200 streptomycete strains for their ability to inhibit the mycelium growth of the target fungi. We observed that some strains strongly inhibited the pathogens, even more than the reference strain *S. lydicus* WYEC 108, the microorganism of the commercial product Actinovate. The production of bioactive compounds with antifungal proprieties plays the major role in the control of pathogens and is a common trait among the bacterial BCAs belonging to *Pseudomonas* spp., *Bacillus* spp, *Serratia* spp. and *Streptomyces* spp. (Behal, 2000; Raaijmakers *et al.*, 2002). A BCA with antibiosis activity can produce numerous secondary metabolites, which have different functions and efficacy against different species of fungal pathogens (Alabouvette *et al.*, 2006). This could explain the different inhibition activity of a single streptomycete strain against various fungi. For instance, *Streptomyces* sp. LAU18R resulted the most active strain against *Sclerotinia sclerotiorum* but did no inhibit the tested Oomycota. Further studies are needed to identify specific secondary metabolites responsible for inhibition of different pathogens.

On the basis of the promising results obtained from the *in vitro* assays, we continued the research focusing the studies on biocontrol of lettuce drop caused by *S. sclerotiorum*, one of the most destructive pathogens of salad crops. Ten streptomycetes, which showed 75% - 90% inhibition activity *in vitro,* were studied in growth chamber and field experiments. Especially, we investigated if both the antagonist application timing in infected substrate and the spore concentration could affect the reduction of disease incidence. When lettuce plants were sown one week after the inoculation of the growing substrate both with the competitor and the pathogen, streptomycetes were found to significantly reduce lettuce drop incidence. *Streptomyces* sp. FT05W, *Streptomyces* sp. SW06W and *Streptomyces* sp. SW29W were the best in reducing the risk of disease incidence by 42%. On the contrary, when lettuce was sown the same day of substrate inoculation no beneficial effect was observed. This suggests that streptomycetes need time to colonize soil before producing antifungal compounds involved in biocontrol and the amount produced could be affected by the abiotic and biotic components of agroecosystem (Raaijmakers *et al.*, 2002). Similar behavior was observed also in other BCAs; *Coniothyrium minitans,* which parasitizes sclerotia of *S. sclerotiorum* is recommended to be applied 8 weeks before sowing of the crop so that sclerotia amount can be considerably lowered and consequently disease incidence (De Vrije *et al.*, 2001; Jones *et al.*, 2004). Similarly, the soil application with *Trichoderma harzianum* and *T. virides* strains one week before crop sowing, was were found to be more effective in reducing the wilt and wet root rot of chickpea, than when applied as seed treatment (Prasad *et al.*, 2002). In our work, spore concentration resulted in significantly different effect on lettuce protection and the effect was strain-dependent.

When we tested the hypothesis that modifying the time of interaction among the antagonist, the host plant and the pathogen could result in a reduction of lettuce drop incidence, we could not get any significant differences in lettuce protection in field-transplanted plants. Since this result is not in agreement with what we found in growth chamber experiment, we speculated that the variability of climatic conditions in field or the complex soil microflora could affect the expression of streptomycete antagonistic activity.

Streptomycetes also showed promising PGP activity because of the frequent production of indole-3-cetic acid (IAA) and siderophores, although they only rarely solubilized phosphate. These traits are very common among the microorganisms that inhabit the rhizosphere (Berg, 2009; Compant *et al.*, 2010; Raaijmakers *et al.*, 2009) and in some works the PGP activity showed *in vitro* was confirmed *in planta*. For instance, rice plants inoculated with siderophore-producing *Streptomyces* sp. enhanced plant growth and significantly increased root and shoot biomass (Rungin *et al.*, 2012).

In field experiments, we investigated if the inoculated strains not only protect lettuce against basal drop, but also enhanced plant growth. However, no significantly PGP effect was obtained assessing the head weights at plant maturity, even though some of the strains used, such as *Streptomyces* sp. CVM02R and *Streptomyces* sp. SW29W actively produced *in vitro* both IAA and siderophores.

To better understand the behavior of a streptomycete strain following its inoculation in soil, we studied lettuce rhizosphere and root tissues colonization with the EGFP labelled strain *Streptomyces* sp. ZEA17I. It showed both rhizospheric and endophytic competences, necessary for its successful biological control activity. Furthermore, we studied the effect of the inoculum type and we observed significantly more colony forming units when the growth substrate was inoculated directly with spore suspension rather than its application through seed coating, and the positive effect was detected both in the plant rhizosphere and in the roots.

According to the results obtained in this study, the bacteria of the genus *Streptomycetes* appear valuable candidates for the biological control of soil borne fungal epidemics. Many interactions between the host plant and the pathogen are still unknown as shown analyzing the contradictory results obtained in different environment, but we think that further studies could provide additional knowledge about their interaction, and can be used to improve the activity of streptomycetes as valuable biological control agents to manage fungi soil borne epidemics.

Materials

Chrome Azurol S agar (Schwyn and Neilands, 1987)

0.0605g CAS, Chrome Azurol S (Fisher Scientific, UK)

72.9mg HDTMA, hexadecyltrimetyl ammonium bromide (Fluka,USA)

30.24 g PIPES, piperazine-1,4-bis-2-ethanesulfonic acid (Sigma-Aldrich, USA)

10 mL 1 mM $FeCl_3$ (Sigma-Aldrich, USA) x $6H_2O$ in 10 mM HCl ()

9 g Agar (Applichem, Germany)

in one liter of distilled water

CZY medium – Czapek Yeast Extract medium

35 g Czapek Dox broth (Difco Laboratories, USA)

2 g yeast extract (Difco Laboratories, USA)

15 g Agar (Applichem, Germany)

in one liter of distilled water

CZY broth - Czapek Yeast Extract broth

35 g Czapek Dox broth (Difco Laboratories, USA)

2 g yeast extract (Difco Laboratories, USA)

in one liter of distilled water

MEA – Malt Extract medium

30 g Malt Extract (Difco Laboratories, USA)

15 g Agar (Applichem, Germany)

in one liter of distilled water

PDA – Potato Dextrose Agar

39 g Potato Dextrose Agar (Difco Laboratories, USA)

in one liter of distilled water

Salkowski reagent

1 mL 0.5M $FeCl_3$ (Sigma-Aldrich, USA)

49 mL 35% $HClO_4$ (Sigma-Aldrich, USA)

Modified Czapek medium for siderophores production

15 g Agar (Applichem, Germany)

30 g Sucrose (Merck, Germany)

1 g K_2HPO_4 (Applichem, Germany)

5 g yeast extract (Difco Laboratories, USA)

10 mL Czapek solution*

in one liter of distilled water.

***Czapek solution (devoid of iron)**

30 g $NaNO_3$ (Carlo Erba Reagents, Italy)

5 g KCl (Merck, Germany)

5 g $MgSO_4$ x $7H_2O$ (Carlo Erba Reagents, Italy)

in 100 mL distilled water.

NBRIY medium - National Botanical Research Institute's phosphate growth medium (Nautiyal, 1999)

10 g Glucose (Applichem, Germany)

5 g $Ca_3(PO_4)_2$ (Sigma-Aldrich, USA)

0.5 g $(NH_4)_2SO_4$ (Carlo Erba Reagents, Italy)

0.2 g NaCl (Carlo Erba Reagents, Italy)

0.1 g $MgSO_4$ x $7H_2O$ (Carlo Erba Reagents, Italy)

0.2 g KCl (Merck, Germany)

20 mg $MnSO_4$ x $2H_2O$ (Carlo Erba Reagents, Italy)

20 mg $FeSO_4$ x $7H_2O$ (Carlo Erba Reagents, Italy)

15 g Agar (Applichem, Germany)

in one liter of distilled water

V8 medium

200 mL V8 Vegetable juice (Campbell food, Belgium)

2 g $CaCO_3$

15g Agar (Applichem, Germany)

in one liter of distilled water

WA - Water Agar medium

15 g Agar (Applichem, Germany)

in one liter of distilled water

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