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Molecular plant-microbiota interactions for biocontrol of plant pathogens

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1 Introduction

In a natural ecosystem, the co-evolution of pathogens and hosts keeps the two in balance: as the plant host evolves defenses against the pathogen, the pathogen will evolve ways to avoid these defenses.

This mechanism keeps diseases from causing severe damage to wild plants, that often appear tolerant to several diseases, and at the same time, prevents the extinction of the pathogen.

In the agricultural ecosystem, crop plants are much more susceptible to the attack by pathogens and pests for several reasons such as the presence of a single, or only a few, plant species attracting specialized pathogens and pests; the sudden introduction of foreign pathogens against which the plant cannot have evolved any defenses; and the selection of genetic traits carried out during domestication that brought wild plants to become the crops we know nowadays, which tended to focus more on the quantity and quality of the produce the crop could yield and, as a result, often ignored defense traits.

For all these reasons, plant diseases are a widespread reality in agriculture, and a serious threat to food security and safety. If left unchecked, some diseases could drastically reduce, even down to zero, the production of crops.

As the knowledge of the etiology and epidemiology of plant diseases increased, it became possible to develop effective methods to control the diffusion of diseases, either by affecting the pathogen directly or by acting on pests that can act as vectors for diseases.

During the previous century, the most effective strategies that were employed to reduce the incidence of diseases were based on the use of chemical compounds, both synthetic (e.g. cyprodinil) or inorganic natural compounds (e.g. copper sulfate), to interfere with the physiology of the pathogen.

While these strategies based on pesticides worked very well in controlling the diseases, they showed several drawbacks, both because of their characteristics and because of improper use, such as the development of resistance to the pesticides from the target organism, side effects on both the agroecosystem and surrounding areas as well as non-target organisms, including people working in the field, living in their vicinity, or eating the crops later on.

These problems became more and more evident as the years passed and, nowadays, the public is very aware and sensitive towards the topic of sustainable agriculture, showing concern towards the safety of the food on their table and the impact that agriculture has on the environment.

This recent sensibility is also reflected in several regulations (such as directive 128/2009 of the European Community) that aim to reduce the use of pesticides to the bare minimum, and completely abolish the pesticides that could have a greater impact on health and the environment.

These laws also increased the pressure towards finding new, alternative strategies to control diseases which could actually be employed in agriculture to substitute these less environmental-friendly practices.

Among the alternative solutions, one that is surely of great importance for its applications and fascinating for scientists is biological control, often shortened as biocontrol.

Biological control is generally defined as the use of a natural antagonist of a detrimental organism to reduce the population, and ability to cause damage, of the undesired organism. In plant pathology, biocontrol is usually intended at a microscopic scale, utilizing beneficial microorganisms, or molecules they produce, to reduce the pathogens' ability to colonize the host or induce symptoms, effectively controlling the disease (Junaid *et al.*, 2013).

Biocontrol offers several advantages over the traditional, synthetic pesticide-based approach, as well as some disadvantages. The main advantage is the specific interaction between the biocontrol agent (BCA) and pathogen which can guarantee a lesser or no effect against non-target organisms and lesser environmental impact as well as reducing the possibilities of developing resistance in the pathogen, as the biocontrol effect is usually determined by several components and a single gene mutation is not enough to negate the effect (Copping and Menn, 2000). At the same time, the disadvantages of working with living organisms are many, including reduced shelf-life compared to conventional pesticides, as well as the unreliable results of their application (Copping and Menn, 2000).

As the ease of use and the success of the treatment are the most important characteristics for successful use in agriculture, research of new BCA, adapted to a different range of plant hosts, climatic conditions, or effective against different pathogens is very important to allow biocontrol to become more and more utilized in the future.

The search for novel BCA often starts from plants that are targeted by the pathogen that the new agent should control: this approach yields candidate BCA that are already adapted to living in association with the crop it will be applied on and already came into contact with the pathogen, having higher chances of being successful as BCA.

Research on the physiology of bacterial BCA is also important for their successful utilization in field, as knowing parameters such as optimal growth parameters, host/target range, and optimal time and quantity for inoculation is paramount for obtaining a good result and a in particular to produce a formulation that can be used successfully in agriculture (Vanneste *et al.*, 2002; Heydari and Pessarakli, 2010).

The two main approaches for applying BCAs is the inundative method – which relies on the massive application of the BCA to obtain an immediate effect, and is comparable to a conventional pesticide treatment (Eilenberg *et al.*, 2001) – or the augmentative method – in which the BCA are applied at a lower concentration, but more often, allowing them to form a stable community which provides a long term effect (Pliego *et al.*, 2011).

Field application of a BCA-based product can follow three methods: the first utilizes an inundative approach, inoculating the infection site with high concentration of the BCA, and it is particularly effective against some fungal pathogen, which results can be in some cases comparable with those obtained with synthetical pesticides (Heydari *et al.*, 2004). A second method, defined ‘one place’, utilizes the augmentative approach, applying the BCA in a localized way and leaving the BCA time to grow and colonize the plant tissues to protect them (Heydari and Pessarakli, 2010). The third method utilizes occasional application of the BCA, usually a microorganism that does not require repeated applications throughout the years, in order to keep the pathogen population beneath the damage threshold (Heydari and Pessarakli, 2010).

1.1 Mechanisms of biocontrol

While many elements of the mechanism underlying biocontrol are still unknown, there are several traits and interactions that have been discovered.

Any one BCA can act on several fronts to exert its biocontrol effect: as disease is the byproduct of the interaction between pathogen, host plant, and the environment, a BCA can act on any of

these elements – or even multiple ones at the same time – to reduce the severity of the disease. If the biocontrol effect is obtained by acting on the pathogen, it is described as being a direct mechanism, while if it acts on the environment or the host, it is described as being an indirect mechanism (Pal and Mc Spadden Gardener, 2006). Examples of direct mechanisms can be parasitism and production of antibiotics, while indirect mechanisms include induction of plant resistance and competition for resources (Compant *et al.*, 2005).

Despite the distinction made between these mechanism, in reality it is hard to discriminate between them as more than one, both direct and indirect, are employed by the BCA (Yedidia *et al.*, 2000). The situation is further complicated by the fact that a BCA could employ different mechanisms based on various factors, such as pH, light, temperature, availability of nutrients, colonized host, and chemical stimuli (Haas *et al.*, 2002). While this variability can become the basis for adaptation to different conditions, it is also part of the reason behind the unreliable results that are often obtained by BCA during field application.

1.1.1 Direct biocontrol mechanisms

The most famous and characterized mechanism of direct biocontrol is the production of antibiotics. An antibiotic is a molecule that can interfere with the development and life cycle of an organism even at very low concentrations. Concentration is a key factor in determining whether a molecule can be considered an antibiotic: for example molecules that are part of the metabolism of many organisms such as ethanol, butanol, and even the amino acid glycin are known to exert a powerful toxic effect on cells, but this toxicity is developed at high concentrations and thus cannot be defined antibiotics (Lancini *et al.*, 1995). These antibiotics are often characterized by low molecular weight (<10 kDalton) and they can have an important role in determining biocontrol efficacy (Thomashow *et al.*, 1997) as it was determined that many BCA can produce antibiotic molecules with a varying degree of specificity towards different pathogens (Raaijmakers *et al.*, 2002).

Many BCA are capable of producing antibiotics that antagonize phytopathogenic microbes and literature can offer interesting examples, such as the production of pyrrolnitrin, pyoluterin, and 2-4-diacethylphloroglucinol by *Pseudomonas protegens* strain Pf-5 (Loper and Gross, 2007). Many Gram-positive bacteria are instead known to produce antifungal lypopeptides, which are often

synthesized through a non-ribosomal mechanism, such as polymyxin produced by *Paenibacillus polymyxa* (Shaheen *et al.*, 2011).

A specific class of antibiotics typical of bacteria is bacteriocins. These antibiotics have a wide range of size and chemical characteristics (Klaenhammer, 1993) but they share their targeted action against other bacteria. Some bacteriocins can affect a broad range of bacteria, while other are very specific and can target different strains of the same species as the bacteria that produces them. Also the mechanism of action of bacteriocins is a characteristic they share, as they produce pores in the protective layers of the target cell, causing imbalance in the concentration of ions and solutes and ultimately the death of the cell (Subramanian and Smith, 2015). While some bacteriocins are reported as being involved in biocontrol, such as the pantocins produced by *Pantoea agglomerans* strain EH318 which are effective against *Erwinia amylovora* (Wright *et al.*, 2001), they are usually limited in their action to strains closely related to the producing BCA and are therefore not versatile molecules (Subramanian and Smith, 2015).

Another important category among antibiotic molecules are some volatile organic compounds (VOCs), characterized by being lipophilic molecules with low molecular weight (below 0.3 kDA) capable of permeating through biological membranes (Abedon, 2015, Giorgio *et al.*, 2015). VOCs have several functions in the physiology of microorganisms and can mediate complex interactions between organisms of the same species or different species, for example being used to regulate quorum sensing and induce resistance (Groenhagen *et al.*, 2013). Among these functions, there is also antibiosis. Several studies reported that VOCs produced by BCA can control rival microorganisms and phytopathogenic fungi and bacteria, suggesting a possible application of these molecules in the future (Audrain *et al.*, 2015; de Vrieze *et al.*, 2015).

The second direct mechanism of biocontrol is parasitism, in which the BCA attacks the pathogen with lytic enzymes to obtain nourishment (Leveau and Preston, 2008). BCA using this mechanism can produce a wide array of enzymes to degrade different molecules, such as chitin, cellulose, hemicellulose, proteins, or DNA in order to degrade the target organism and kill it to obtain nutrients (Ordentlich *et al.*, 1988; Junaid *et al.*, 2013). Chitinase-producing strains of *Bacillus* and *Pseudomonas* were reported as being capable of reducing the development of pathogenic fungi (Yu *et al.*, 2008), but these mechanisms are often employed only when the BCA faces starvation and needs to rely on the pathogen for sustenance. For example, many fungi of the *Trichoderma*

genus employ their efficient lytic systems to degrade pathogenic fungi only when they face cellulose starvation (Altomare *et al.*, 1999).

1.1.2 Indirect biocontrol mechanisms

Pathogens are often very specialized organisms that have to establish specific interactions with their hosts in order to complete their vital cycle and thrive. These interactions must allow them to obtain nutrients from the host and this can often happen only if the pathogen can reach certain parts of the host. These interactions can be hindered if another organism competes for the limited resources available within the host or for the colonization of the same environment; this competition for nutrients and space can be a mechanism of biocontrol (Pal and Mc Spadden Gardener, 2006).

As both nutrients and space are limited resources, both inside a plant and in the soil, the successful development of a BCA can hinder or negate the growth of a pathogen competing for the same resources. A common resource that is object of fierce competition among microorganisms in soil and plant tissues is iron: this microelement is usually found in its ferric, insoluble form in soils and its concentration can greatly limit the development of microorganisms. Given this situation, the microorganisms that have more efficient iron-uptake mechanisms, often mediated by chelating oligopeptides called siderophores, will prevail over less efficient competitors, determining inhibition of development (Nelson, 1990). Also related to this specific mechanism of competition is the hypothesis of some BCA employing a 'siderophore parasitism' approach: these microorganisms would be able to uptake the iron chelated by siderophores produced by their competitors, obtaining iron at the expense of the microorganisms who produced the chelating agents in the first place (Mitter *et al.*, 2013).

Competition for space can be used to prevent the colonization by a pathogen by allowing a non-pathogenic organism to occupy the interface the pathogen requires to enter in the host. To be efficient, the BCA must grow at a higher rate than the pathogen in that same ecological niche (Maloy, 1993). A recent of example of this mechanism being used in biocontrol is the protection of *Pinus* trees stumps, in which the fungus *Phlebiopsis gigantea* is inoculated in the cut area in order to colonize the wound before *Heterobasidium annosum*, a pathogenic fungus, can. As this pathogen spreads from the stumps to healthy plants that still have to be cut down, protecting the stumps prevents the spread of the disease (Mesanza *et al.*, 2016). Production of biofilm can also

be effective for negating a pathogen access to a host, as was demonstrated for the yeasts *Aureobasidium pullulans* and *Wickerhamomyces anomalus*, which prevented the pathogen *Botrytis cinerea* from colonizing wounded fruits after developing a film on their surface (Parafati *et al.*, 2015).

Another interesting aspect of indirect biocontrol is the way in which a BCA can induce resistance in a host plant, allowing the plant to better resist pathogen attacks (Junaid *et al.*, 2013).

In order to conserve energy, plants have evolved systems that allow them to recognize whether the microorganisms they come in contact with are beneficial, indifferent, or pathogenic, and they activate defense mechanisms only when they recognize a microorganism as a pathogen, since these defenses require a high metabolic toll to be activated (Compant *et al.*, 2005; de Leon and Montesano, 2013).

When a plant recognizes an attack, it activates its defense systems, having greater possibilities of thwarting the pathogen the quicker the response is (Jain et al, 2012). The defense systems of a plant are often aspecific and are therefore effective against a wide range of pathogens and pests, and they are typically divided in: local acquired resistance (LAR), systemic acquired resistance (SAR), and induced systemic resistance (ISR) (Sarma *et al.*, 2015).

LAR and SAR are defensive mechanisms that are activated in response to pathogen attack, are mediated by salicylic acid, and induce the expression of pathogenesis-related proteins (PR). These proteins have several functions that include the lysis of pathogen cells, reinforcement of cell walls and programmed cell death. ISR is a separate phenomenon which is not activated by lesions or cell death, is mediated by jasmonic acid and ethylene.

Some molecules of microbial origin, such as lipopolysaccharides or flagellin, can bind to plant receptors and activate ISR (Shi *et al.*, 2011). These molecules can originate from beneficial bacteria that can act as biocontrol agents by activating this mechanism of plant defense. Some well-known BCA can activate ISR in a plant host, such as *Burkholderia phytofirmans* strain PsJN that has been demonstrated to activate resistance in grapevine and tomato plants, and *Bacillus pumilus* strain SE34 in some leguminous plants (Compant *et al.*, 2005). It is also reported that some VOCs produced by bacteria can mediate this process of inducing resistance in plants (Sharifi

and Ryu, 2016), broadening the range of molecules that can activate this mechanism, and the ways in which bacteria can stimulate plant defenses.

1.2 Microbiota and biocontrol

Until a few decades ago it was believed that plants that did not show symptoms were sterile environments, now it is common knowledge that every plant hosts entire communities of microorganisms, defined as their microbiota. This microbiota is made up of different species of microorganisms, some of which can be beneficial, some indifferent, and some pathogenic. The microbiota is so important in determining the physiology and life of the plant that the whole array of genes and functions that these microorganisms possess – called microbiome – is essential to allow the plant to adapt to different environments and changes (Bulgarelli *et al.*, 2013). The interaction between a plant and its microbiome is so tight that the system of plant host and microbiota can be viewed as a plant holobiont (Zilber-Rosenberg and Rosenberg, 2008).

As the importance of the microbiome is becoming more and more clear, several studies were carried out to identify how the microbiome is influenced by diseases (Trivedi *et al.*, 2010, Bulgari *et al.*, 2011, Larousse *et al.*, 2017). These studies describe the difference in the taxon present in plants affected by specific diseases and healthy plants, identifying shifts in microbial communities which can be interesting markers for the sanitary status of plants, defining the characteristics of a healthy microbiome, and even specific strains or species that are unique to healthy plants.

The complexity of the microbiome of a plant, based on several interactions between microorganisms and the host, as well as with one another, is extremely difficult to analyze as a whole. In recent years newer tools are becoming available to investigate these complex microbial communities, thanks to the advances in molecular microbiology and in sequencing techniques (Bai *et al.*, 2015). For example, high-throughput sequencing of the microbiota allows to 'take pictures' of the situation of the microbial community inside a plant and, by comparison of the different 'shots', deduct which components of the microbiota can be related to sanitary status. Seeing how recent studies highlighted similarities between human gut microbiota and plant rhizosphere microbiota (Ramirez-Puebla *et al.*, 2013, Berg *et al.*, 2015, Hacquard *et al.*, 2015), and how medical use of microbiota transplant is giving promising results (Kelly *et al.*, 2014), it is not

impossible that also the health of a crop could be restored or preserved by inducing a shift in its microbiota by applying specific strains or consortia of microorganisms to it, either for its own biocontrol effect, or by activating the defense potential in the host's existing microbiome (Podolich *et al.*, 2015).

For this reason, in the latest years, many studies were carried out on endophytic BCA that can effectively colonize the plant host and, possibly, help maintaining the whole microbiota of the host in a healthy condition, as well as providing direct beneficial effect for the host (Ryan *et al.*, 2008; Compant *et al.*, 2010).

1.3 Bacterial biocontrol agents

Many BCA-based products employ fungi of the *Trichoderma* genus as active microorganism, but there are several commercial products that rely on bacteria to control diseases and/or promote plant growth. This mixed category is given by the fact that, as the distinction between a biocontrol and plant-growth promoting bacteria is often blurred, it is difficult to separate clearly products that promote plant growth from those that offer protection against pathogen. The bacteria that found employment in these products are known mostly to belong to the genera (or species) *Agrobacterium*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Delfitia*, *Paenibacillus macerans*, *Pantoea agglomerans*, *Pseudomonas*, *Rhizobium*, and *Serratia*; among which the two most utilized are surely *Bacillus* and *Pseudomonas* (Berg, 2009; Glick, 2012).

The success of these two genera is based on the great background of knowledge that is available on the modes of action and interaction with hosts that these bacteria establish with plant hosts in laboratory, greenhouse, and field conditions. Strains of *Bacillus subtilis*, *Bacillus pumilus*, *Pseudomonas fluorescens* and *Pseudomonas protegens*, for example, have extensive literature describing their biocontrol potential and mechanisms since decades ago (Shoda, 2000; Santoyo *et al.*, 2012). In recent years also several strains belonging to the genera *Azoarcus*, *Exiguobacterium*, *Methylobacterium*, *Paenibacillus*, and *Pantoea* are being characterized as interesting for their biocontrol potential (Chauhan *et al.*, 2015).

Another class of bacteria which is interesting for biocontrol are beneficial endophytic bacteria. These bacteria have the advantage of being stably associated with their host plant and can have the

beneficial effect typical of BCA (Hallmann *et al.* 1997; Kloepper *et al.* 2004). Furthermore, endophytic bacteria that are naturally associated to a plant species are more likely to offer better control of some diseases and promotion of growth compared to organisms isolated from other plant species (Long *et al.* 2008), making them valuable while researching novel BCA effective against particular diseases .

While new species and strains showing biocontrol potential are very promising in laboratory and greenhouse studies, strict safety controls must be undertaken to assess the potential risks involved with their use in agriculture. The main concern with the utilization of BCA is for the impact on the environment and for human health, both in the scope of their intended use and in case of accidental, unregulated release in the environment. The effect on human health is a factor that has to be considered, especially for bacteria of genera that are known to be related to human pathogens, such as *Pantoea* and *Burkholderia*.

Genomic-level studies, aimed at identifying the traits related to pathogenesis to assist screening of human pathogens while selecting BCA, is of particular importance for strains and species of the genus *Burkholderia*, which includes many plant beneficial microorganisms that are pathogenic for animals and humans in the subgroup defined as *B. cepacia* complex (Paganin *et al.*, 2011).

Also because of these concerns for health, describing a new BCA at a genome level is an obligatory step in registration of a microbe for commercial use, and genomic studies on BCA, aimed at characterizing common genes at the basis of their effect or identify the genes that distinguish between pathogenic and beneficial bacteria are becoming more and more common (Bloemberg and Lugtenberg, 2001).

At the same time, genomic analyses are becoming more easily available and accessible, allowing to obtain more genome data at a quicker pace, and in turn to gain more precise insights on the molecular mechanisms underlying complex biological processes (Collins *et al.*, 2003).

In the field of biocontrol, a main goal related to genome analysis is to identify genetic markers that can allow to distinguish from the genome alone which bacteria could have potential as BCA, and which could be potential pathogens for plants or animals. This question is still far from being resolved and the current theories are very diverse: the similarity between closely related pathogens and helpful bacteria is suggesting that, in many cases, it is not the presence or absence of specific

genes that determines the difference between pathogenic and beneficial behavior, but rather a difference in the expression of the genes (Sheibani-Tezerji *et al.*, 2015). Another possibility is that the differences lie in the plethora of genes which function is still unknown, and that are therefore impossible to analyze through genomics alone.

2 Aim of the Study

In previous studies, the bacterial community associated with plants characterized by different sanitary status in regards of phytoplasmatic diseases was investigated. In particular, these studies focused on (i) grapevine plants that were either healthy, infected by phytoplasma associated with flavescence dorée disease, or recovered from flavescence dorée (Bulgari *et al.*, 2011), and (ii) apple trees that were either healthy, or infected by phytoplasma associated with apple proliferation disease (Bulgari *et al.*, 2012; Spreafico 2012-2013).

These studies brought to the isolation of several bacterial strains that, based on their taxonomy, presence in non-diseased plants, and preliminary biochemical assays, showed potential as biocontrol agents. In particular, the hypothesis that these strains could be involved in the ability of the plant to resist the infection by phytoplasma made the analysis of these strains interesting, as it could possibly contribute to finding novel, sustainable way to control systemic diseases against which traditional control methods are not effective.

In this study, 7 of these strains were investigated to determine their ability to antagonize the growth and/or ability to develop symptoms of phytopathogens (bacteria, fungi, and viruses) and to promote plant growth through several assays carried out at different levels (*in vitro*, *in vivo*, *in planta*). While these strains were identified in relation with phytoplasmatic diseases, they were assayed against various kinds of phytopathogens, both to determine if they had a broad range of antagonism, and to work with a simpler model compared to phytoplasma-host interaction.

The 7 strains used in this work have a varied taxonomic background, with the strains isolated from apple trees belonging to the species *Pantoea agglomerans* (strain 255-7 and the transformed strain 255-7:*rfp*), *Burkholderia* sp. (strain 255-8), *Pseudomonas syringae* (strain 260-02 and the transformed strain 260-02:*gfp*), and *Lysinibacillus fusiformis* (strain S4C11); while the strains isolated from recovered grapevine plants belong to the species *Bacillus pumilus* (strain R1), *Burkholderia* sp. (strain R8 and the transformed strain R8::*gfp*), and *Paenibacillus pasadensis* (strain R16).

The *in vitro* assays had the aim of determining if the strains could inhibit the mycelial growth of different phytopathogenic fungi when co-cultured, or with the exclusive use of diffusible or

volatile molecules, which were characterized. Furthermore, the ability to inhibit the germination of conidia of *Botrytis cinerea* was investigated during this assay.

The *in vivo* assays had the aim of determining if the strains were capable of reducing the infection rate of *Botrytis cinerea* on detached organs (leaves, fruits) of host plants (grapevine, tomato).

The *in planta* assays had the aim of determining if the strains could promote plant growth and reduce the symptoms induced by a pathogenic fungus (*Rhizoctonia solani*), bacterium (*P. syringae* pv. tomato), and virus (*Cymbidium Ringspot Virus*).

Another goal of the study was to investigate not just the presence of these effects, but offer insight on the mechanisms underlying these effects, in particular regarding the interactions between the selected strains, the pathogens, and the host plants. To this aim some assays were carried out to determine the colonization of the strains in a range of host plants, ultimately focusing on pepper as the main plant used in the study, and the effect of the colonization on the methylation pattern and gene expression when the plants were challenged by the virus.

These assays were accompanied by genomic analyses carried out on the seven strains, to assess the genetic background of these candidate biocontrol agents and, in particular for strain 260-02, identify genes that might be essential for beneficial interaction with a plant host.

The obtained results will allowed to identify which bacterial strains show better characteristics as biocontrol and plant-growth-promoting agents, as well as give insight in the mechanisms of action and interaction at the base of their effects.

3 Materials and Methods

3.1 Materials

3.1.1 Candidate bacterial Biocontrol Agents (BCA)

In the present study, 7 putative endophytic bacterial strains were examined:

- Strain 255-7, *Pantoea agglomerans*
- Strain 255-8, *Burkholderia* sp.
- Strain 260-02, *Pseudomonas syringae*
- Strain S4C11, *Lysinibacillus fusiformis*
- Strain R1, *Bacillus pumilus*
- Strain R8, *Burkholderia* sp.
- Strain R16, *Paenibacillus pasadenensis*

Strains 255-7, 255-8, 260-02, and S4C11 were isolated from the roots of apple plants in a previous work, while strains R1, R8, and R16 were isolated from leaves of grapevine plants in a previous work.

In particular, the apple plants were sampled in two consecutive years, March 2011 and March 2012, as part of a survey on the phytoplasmatic disease apple proliferation carried out in the orchard of the Minoprio Foundation, in the Vertemate con Minoprio town (CO). These strains were recovered exclusively from healthy plants (Bulgari *et al.*, 2012, Spreafico, 2012-2013).

The grapevine plants were sampled in the years between 2006 and 2009 from a Barbera cultivar vineyard in the Oltrepò pavese area, in the province of Pavia, as part of a study on *flavescence dorée* and recovery from this disease (Bulgari *et al.*, 2011). The grapevine plants from which all three strains were isolated were confirmed to have underwent recovery, showing a remission of symptoms from at least 3 years, accompanied by a non-detectability of the phytoplasma responsible for the disease in these plants.

The strains were chosen from a larger collection obtained from the studies mentioned above, after having been characterized on the following traits related to plant-growth promotion and/or biocontrol, as well as kanamycin resistance to determine if the strains could be selected by this antibiotic and were suitable hosts for plasmids carrying this resistance:

- Phosphate solubilization, using Pikovskaya medium (Pikovskaya 1948) with insoluble $Ca_3(PO_4)^2$ added (5 g/liter)
- Chitinase production, using the medium reported by Sridevi and Mallaiah (2008)
- Siderophore production, using CAS agar medium as described by Alexander and Zuberer (1991)
- ACC-deaminase activity, using minimal DF medium (Dworkin and Foster, 1958) prepared as described by Hunsen *et al.* (2009)
- Catalase activity, tested by dripping 3% hydrogen peroxide directly on the bacterial colonies
- Production of indole acetic acid, using the method described by Pilet and Chollet (1970)

Results of these assays are presented in **Table 1**.

All the chosen strains were maintained on LBA (see Appendix) plates and grown at 24 °C for routine propagation and short-term conservation, and were stored at -80 °C in an LB:glycerol (7:3) solution for long-term storage.

Some of these strains (255-7, 260-02 and R8) were successfully marked by labeling with a fluorescent protein (Red Fluorescent Protein for 255-7 and Green Fluorescent Protein for 260-02 and R8) by electroporation of a replicative (pRL765rfp in strain 255-7, pHM2-GFP in strain 260-02) or integrative plasmid (pUTgfp2x in strain R8). All the utilized plasmids carried resistance against kanamycin as a selection marker, and the fluorescent protein gene allowing recognition of the strain during colonization assays, later carried on *in planta* (see 3.2.3.1). These mutants are identified as 255-7:*rfp*, 260-02:*gfp*, and R8::*gfp*, respectively.

Table 1 Summary of the biochemical characterization performed on the 7 examined strains. In different rows are presented different assays, while different columns report the results for different strains. A + indicates a positive result for that function, while a – indicates a negative result for that function.

	255-7	255-8	260-02	S4C11	R1	R8	R16
Siderofore production	-	+	+	+	-	+	-
Phosphate solubilization	+	-	+	-	+	+	-
ACC deamination	-	-	-	-	+	+	-
IAA production	+	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	+	+
Chitinase activity	-	-	-	-	-	-	+
Kanamycin resistance	-	+	-	-	-	-	+

3.1.2 Phytopathogenic agents

To test the biocontrol efficacy of the selected strains, they were assayed against several phytopathogenic agents of different natures: fungi, bacteria, and viruses.

The phytopathogenic fungi used in this study are:

- *Aspergillus* sez. *nigri*, strain AsN1 (which will be identified as AN for the rest of the study), isolated in 2015 from rotting grape berries (*Vitis vinifera* L.).
- *Botrytis cinerea* Pers. strain MG53 (which will be identified as BC for the rest of the study), isolated in 2014 from wheat (*Triticum aestivum* L.) kernels
- *Fusarium verticillioides* (Sacc.) Nirenberg, strain GV2245 (which will be identified as FV for the rest of the study), isolated in 2011 from an ear of corn (*Zea mais* L.) that showed symptoms ascribable to pink fusariosis
- *Phomopsis viticola* (Sacc.) Sacc., strain PV1 (which will be identified as PV for the rest of the study), isolated in 2012 from berries of grapevines (*V. vinifera*) which showed black rot symptoms
- *Rhizoctonia solani* (Cooke) Wint, strain RS1 (which will be identified as RS for the rest of the study), isolated from millet (*Pennisetum glaucum* L.) kernels in 2012

All these fungi were obtained from the mycology laboratory of the Biodefense Unit of the Faculty of Agricultural and Alimentary Sciences of the University of Milan. These fungi were stored for short periods and grown routinely on PDA (see Appendix) plates at 20 °C, and stored for longer periods at 4 °C in glass tubes containing a layer of PDA medium.

In this study, a single phytopathogenic bacterial strain was utilized, *Pseudomonas syringae* pv. *tomato* DC3000 (obtained from a collection of University of Natural Resources and Life Sciences of Vienna). This strain was kept in the same growing conditions as the biocontrol agents and, also for this strain, a fluorescent mutant with integrated pUTgfp2x plasmid, carrying *gfp* and kanamycin resistance genes. This mutant is identified as DC3000::*gfp*.

Furthermore, a phytopathogenic virus was used in biocontrol assays, an isolate of *Cymbidium Ringspot Virus* (CymRSV), bought from the Deutsche Sammlung von Mikroorganismen und

Sellkulturen GmbH (DSMZ) and periodically inoculated on *Nicotiana benthamiana* plants, which infected leaves were sampled and stored at -80 °C to be used as inoculum source for the virus.

3.1.3 Plant material

During this study, several different plants, or part thereof, were used as systems to inoculate pathogens, biocontrol agents, or both.

In particular, the following plants were grown from seed in the greenhouse of the department:

- *Capsicum annuum*, var. Quadrato d'Asti (Which will be referred to as Quadrato d'Asti for the rest of the study)
- *Capsicum annuum*, Zebo F1 hybrid (which will be referred to as Zebo for the rest of the study)
- *Cucumis sativum*, Burpless Tasty Green F1 hybrid (which will be referred to as Tasty Green for the rest of the study)
- *Cucurbita pepo*, var. Genovese (which will be referred to as Genovese for the rest of the study)
- *Nicotiana benthamiana*
- *Nicotiana tabacum*
- *Phaseolus vulgaris*, var. Nano Bobis sel. Monica (which will be referred to as Nano bobis for the rest of the study)
- *Solanum lycopersicum*, var. Rio grande (which will be referred to as Rio grande for the rest of the study)
- *Solanum lycopersicum*, var. San Marzano 2 (which will be referred to as San Marzano for the rest of the study)
- *Solanum lycopersicum*, Sibari F1 hybrid (which will be referred to as Sibari for the rest of the study)

- *Solanum melongena*, Beatrice F1 hybrid (which will be referred to as Beatrice for the rest of the study)
- *Solanum melongena*, Violetta lunga F1 hybrid (Which will be referred to as Violetta for the rest of the study)

The following detached plant parts were used throughout the study:

- *Solanum lycopersicum*, cherry tomato berries, bought from a local grocery
- *Vitis vinifera*, var. Red Globe leaves, detached from potted plantlets obtained from micropropagation (bought from Vitroplant Italia Srl)

3.1.4 *In silico* analyses

In this study, specialized software was used for some analyses of the numerical data gathered from experiments, and to analyze genomic data.

All statistical analyses of data described in the study were performed using IBM SPSS v. 24 statistic package.

The Illumina and MinION reads obtained in order to sequence the genomes of the strains were corrected, assembled into genomes, and the genome data were analyzed and compared using the following software: Sickle (Joshi and Fass, 2011), BayesHAMMER (Nikolenko *et al.*, 2012), MinKNOW, SPAdes (Bankovich *et al.*, 2012) 2.9.0, RAST (Aziz *et al.*, 2008), Blast2GO (Conesa *et al.*, 2005), antiSMASH 3.0 (Weber *et al.*, 2015), PROKKA (Seemann, 2014) Roary (Page *et al.*, 2015).

3.2 Methods

3.2.1 *In vitro* biocontrol assays

All assays carried out *in vitro* to test the ability of the 7 bacterial strains to control fungal pathogens will be described in this chapter.

3.2.1.1 Dual-culture assay

Antagonism studies by dual-culture plate method were carried out on TGYA (see Appendix) plates, since some bacterial strains had difficulties growing on PDA medium, and some fungal strains had difficulties growing on LB medium (data not shown). On each plate four 0.5 cm discs of sterilized filter paper were placed at approximately 4 cm from the center of the plate and evenly distributed on its surface. On each disc was placed a 20 µl drop of overnight culture of one bacterial strain (see Appendix), diluted so that each 20 µl drop contained 2 $x10^5$ CFUs . After inoculation of the bacteria, the plates were incubated for 2 days at 25 °C in the dark before proceeding with fungal inoculation. The fungal inoculum was a 0.5 cm in diameter mycelial plug excised with a sterilized cork borer from the edges of an actively growing fungal culture placed in the middle of the TGYA plate (**Figure 1**). This test was carried out using all 7 bacterial strains against the phytopathogenic fungi AN, BC, FV, PV, and RS. As negative control, plates containing the fungal strains alone were used.

Fungal growth was measured 5, 7, and 14 days post inoculation (dpi) as mycelial growth diameter. Each test was carried out with plates in triplicate and three independent measures were made for each plate at each measuring time. Growth inhibition percentage (GIP) was calculated as [1-(D1/D2)]x100, where D1 is the radial colony growth on bacteria-treated plate, D2 is the radial colony growth in the control plate.

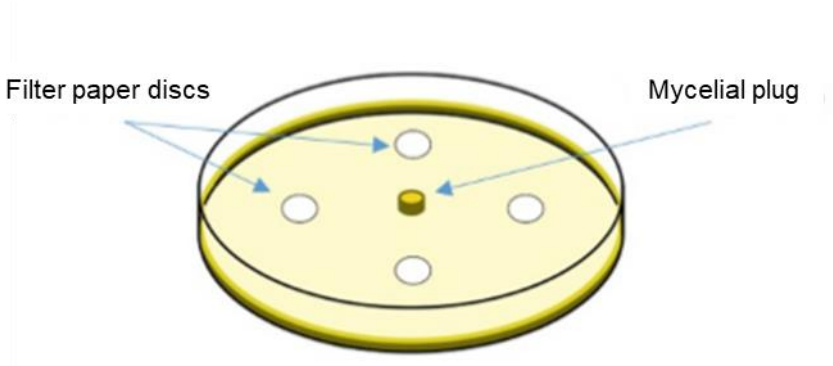


Figure 1 Schematic representation of the setup used to perform dual-culture inhibition assays, showing the layout of the fungal inoculum (mycelial plug) and supports used for bacterial inoculations (filter paper discs) inside the Petri dish.

3.2.1.2 Dual-plate assay

To evaluate the strains' ability to inhibit fungal growth through the production of volatile compounds, a dual-plate assay was carried out as described by Chaurasia *et al.* in 2005. Briefly, 100 µl of overnight culture in LB of each strain (see Appendix), diluted to 10^7 CFU/ml, was diffused on the surface of a TGYA plate and then incubated overnight at 25°C. After two days, a 0.5 cm in diameter, fungal mycelial plug was inoculated onto another TGYA plate, then the cover of the plate with the bacteria was replaced with the upturned plate containing the fungal culture under sterile conditions, and the plates were sealed together with Parafilm. Negative controls were obtained by putting a plate containing the fungal inoculum over a plate containing only TGYA, and no bacterial inoculum. This test was carried out using all 7 bacterial strains against the phytopathogenic fungi BC, FV, and PV. After fungal inoculation, all the plates were kept at 25°C in the dark and the fungal growth was measured 14 dpi. Each test was made with plates in triplicate and three independent measures were made for each plate. Growth inhibition percentage determined by volatile compounds (GIPv) was calculated using the same formula indicated for GIP.

3.2.1.3 Characterization of volatile compounds produced by candidate BCA strains

After having determined that all examined strains had the ability to synthesize volatile organic compounds (VOCs) capable of inhibiting fungal growth, the exact composition of their volatile bouquet was determined.

Analysis of VOCs produced by the 7 strains was carried out using a method which reproduced the dual-plate assay on the inside of a head-space crimp-top glass bottle (10 ml, Agilent, Santa Clara, CA). Inside the sterilized glass bottle, two layers of TGYA (prepared as described before, except using 3% agar) were prepared on opposite sides, making sure that the layers did not touch one another, allowing to inoculate the different microorganisms on either layer without coming into contact with the other one (**Figure 2**). Furthermore, the collection fiber could be inserted directly in the bottle, without coming in contact with the solid media or the microorganisms, but only with the volatile compounds present in the gas layer between the two. To test which volatiles were produced by the bacterial strains and which were produced by BC, the following vials were prepared: (i) bottles in which only the bacterial strain was growing, (ii) bottles in which only BC was growing, (iii) bottles in which both the bacterial strain and BC were growing, and (iv) bottles

with only TGYA medium, to be used as negative controls. The inoculation of both fungus and bacteria was carried out on the same day for these trials, to avoid losing VOCs by opening the bottle before the analysis. The bottles, prepared in triplicates, were then closed with their cap and sealed with Parafilm, and left in incubation at 25 °C in the dark for 14 days before VOCs analysis.

The analysis was performed using the Solid-Phase-Micro-Extraction technique followed by Gas Chromatography-Mass Spectrometry (SPME-GC-MS). VOCs were adsorbed on a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fiber and desorbed at 260°C in the injection port of an Agilent Technologies 6890N/5973N gas chromatograph-mass spectrometer equipped with a 60 m × 0.25 mm × 0.25 μm 100% polyethylene glycol column (Zebron ZB-WAX plus, Phenomenex). Helium was used as the carrier gas in constant pressure mode (150 kPa). The oven was programmed at 45 °C (5-min hold) and ramped up to 219 °C at 6 °C/min (16-min hold). The transfer line to the mass spectrometer was maintained at 280 °C, while the ion source was 230 °C, and the quadrupole was 150 °C. Acquisition was performed in electronic impact mode. VOCs were identified using the Wiley 7n-1 MS library on Agilent MSD ChemStation software (Agilent Technologies Inc.). Confirmation of the identity of the volatile compounds was achieved by comparing the GC retention indices and mass spectra of individual components with those of authentic reference compounds (Sigma-Aldrich Co.) under the same operating conditions.

The data collected refer to the peak area of the quant ion of each compound, and are expressed as $\log_{10}(Area + 1)$, for this reason the determination must be considered semi-quantitative. Compounds were considered "absent" (i.e. not produced) when the signal to noise ratio was below 2:1. To check the presence of carry-over effects, blank extractions were conducted regularly.

The peak areas for each compound were, when present in more than one treatment, compared between treatments by performing one-way ANOVA, followed by Tukey's exact post-hoc test ($p < 0.05$)

This SPME-GC-MS analysis was carried out in collaboration with Dr. Giovanna Battelli at the National Council of Research (CNR).

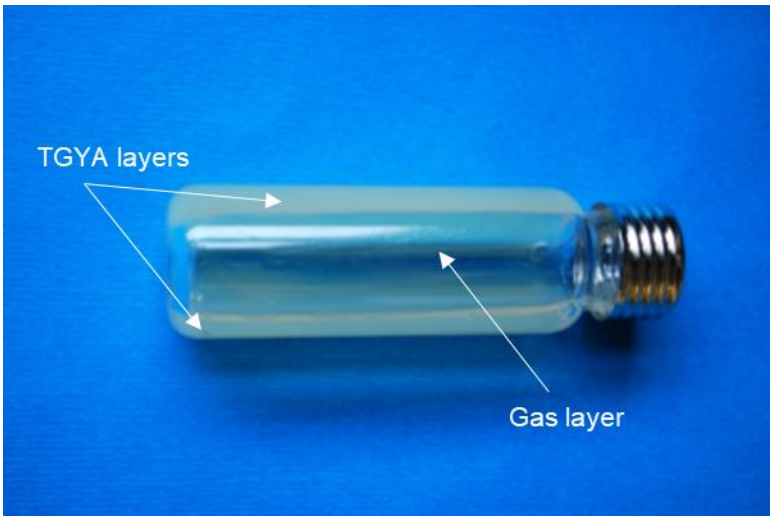


Figure 2 Picture showing the setup of the glass bottles used during the SPME-GC-MS analyses, describing the position of the two agar medium layers (TGYA layers) and the empty space between them (gas layer) from which the volatile compounds have been extracted.

3.2.1.4 Inhibition of *B. cinerea* myceliar growth by cultural filtrate

The ability of the strains to inhibit myceliar growth of BC through the production of diffusible molecules was carried out by inoculating the fungus on a sterile agarized medium which was made of 50% TGYA 2x (see Appendix) and 50% by the solution obtained by filter-sterilizing the culture broth of each strain.

This culture broth was obtained by pre-inoculating a single colony of each strain in a 50 ml sterilized tube containing 10 ml of LB and incubating it at 25 °C overnight with shaking (150 rpm). After this pre-inoculation, 1 ml was taken from each tube and inoculated in a 500 ml conical flask containing 100 ml of LB, incubated at 25 °C with shaking (150 rpm) for 3 days. After 3 days of incubation, the culture broth was centrifuged to pellet the cells (8000x *g* for 10 minutes) and the supernatant was vacuum filtered (pore diameter 0.22 µm) to eliminate possible cellular residues.

After the agarized medium solidified, the plates were incubated overnight at 25 °C in the dark to make sure that no living cells were still present in the medium before proceeding with fungal inoculation. The fungal inoculum was a 0.5 cm in diameter mycelial plug excised with a sterilized cork borer from the edges of an actively growing BC culture placed in the middle of the plate. This test was carried out using the filtrate of all 7 bacterial strains against the phytopathogenic fungus BC. As negative control, plates prepared using TGYA without added bacterial culture filtrate, inoculated with BC were used.

Fungal growth was measured 7 dpi as mycelial growth diameter. Each test was carried out with plates in triplicate and three independent measures were made for each plate at each measuring time. Growth inhibition percentage obtained with the filtrates (GIPf) was calculated as described previously.

3.2.1.5 Inhibition of spore germination of *B. cinerea* by co-culture

The bacterial strains' ability to reduce the germination of conidia produced by BC when both the conidia and the bacterial cells were present in the same solution was evaluated. In this assay, solutions of freshly harvested BC conidia, with a final concentration of 10^4 conidia/ml, were tested for germination in TGY medium (see Appendix) either alone, or in the presence of each strain. Conidia were harvested by pouring 5 ml of sterile water added with Tween™ 20 (0.01%) to a BC

plate with visible production of conidia over the mycelium and scraping the plate with a sterile loop; the obtained solution was then filtered through a double layer of sterile gauze to remove larger pieces of mycelium. This solution was serially diluted and the quantity of conidia per ml was evaluated in each dilution by direct count using optical microscopy and a KOVA plate (Hycor Biomedical Inc., USA) and then adjusted to the desired concentration. The bacterial strains were added at a final concentration of approximately 10^5 CFU/ml, and the assay was carried out in 2 ml tubes, using a total volume of 1 ml. Both the treated samples and non-treated controls were set up in triplicates. The tubes were incubated for 72 hours on an orbital shaker, kept in the dark at a temperature of 25 °C, and germination rates were measured in triplicate after 6, 18, 24, 48, and 72 hours of co-culture. Germination was evaluated by direct observation under an optical microscope (20X; Easylab CX40, Olimpus) using a Kova counting grid, considering each spore to have germinated if the length of germination tube was twice as long as the conidium diameter (Chen *et al.* 2008). For each observation, 100 spores were visually analyzed and determined to be either germinated or non-germinated. Conidial germination rate (GR) was calculated as (G/C)x100, where G is the number of germinated conidia detected, and C is the total number of conidia counted. The visual examination was also confirmed with the spectrophotometry method reported by Raposo and colleagues in 1995 (data not shown).

The germination rate measured in this assay was then compared between the various treatments through one-way ANOVA, optimized for repeated measures, applying Tukey's exact post-hoc test ($p < 0.05$).

3.2.1.6 Inhibition of spore germination of *B. cinerea* by cultural filtrate

Like for the inhibition of myceliar growth, the ability of the culture broth filtrate to inhibit the germination of BC conidia was evaluated.

The cultural filtrate was obtained as described above and added at a 50% v/v concentration to a BC conidia suspension in TGY 2x (final concentration 10^4 conidia/ml). Negative control was obtained by adding 50% v/v of sterile LB medium instead of a cultural filtrate to the conidia suspension.

The obtained solution was then placed in a 96-wells polystyrene plate (Sero-Wel™, Bibby Sterilin), 200 µl per well and making 7 replicates for each strain. The plate was then incubated at

20 °C in the dark for 48 hours, and germination of conidia was evaluated at the beginning of the experiment and again after 6, 12, 18, 24, 36, and 48 hours by measuring the absorbance at 492 nm wavelength using a plate reader (Sunrise™ Absorbance Reader, Tecan Ltd Mantendorf) (Raposo *et al.* 1995). From the visual inspection of conidia germination described in the previous paragraph, we could determine that the germination of conidia happened prevalently during the first 24 hours in the non-treated controls, while at later times the increase of absorbance was mostly determined by the development of mycelium. Therefore, absorbance data obtained after the first 24 hours of incubation were not used to determine inhibition, but only to confirm whether the effect detected in the first 24 hours was maintained, or diminished over time. To keep data obtained from the first 24 hours comparable to those obtained over the following 24 hours, the results will be expressed as raw absorbance, rather than a percentage of inhibition.

Furthermore, to try and characterize which kind of components could determine the inhibition effect on conidia germination, the assay was repeated using a cultural filtrate that was either treated with proteinase K (200 μg/ml; 37 °C for 30 minutes), degrading proteins, or subjected to a heat shock by autoclaving at 121 °C for 20 minutes. These assays could determine whether proteins or thermosensitive molecules are involved in the inhibition process. In these assays, the first reading of absorbance was performed after 12 hours from incubation, having determined that before that time no germination of conidia was detectable in these experimental conditions.

Absorbance data obtained in these assays was then compared between the various treatments through one-way ANOVA, optimized for repeated measures, applying Tukey's exact post-hoc test ($p < 0.05$).

3.2.2 *In vivo* biocontrol assays against *B. cinerea*

All assays carried out *in vivo* to test the ability of the 7 bacterial strains to control BC infection will be described in this chapter. In this study, we consider experiments carried out on living plant organs detached from the organism (e.g. fruits or leaves) as *in vivo* assays, as reported in previous works (Fernandez-Ortuno *et al.* 2013, Haidar *et al.* 2016).

3.2.2.1 Assays on tomato berry

These assays were carried out on detached plant material: cherry tomato berries, as described in Materials. Tomatoes of uniform size were surface sterilized by washing in the sterilizing solution

for 5 minutes, and then rinsed three times in sterile water to wash away all residues of the solution. After the sterilization, the berries were dried under laminal flow hood before being dipped in a bacterial solution of each strain (10^5 CFU/ml) for 12 seconds and then left to dry under laminal flow hood.

After drying, 4 wounds were made on the equatorial area of each berry using a sterile needle. In each of these wounds 20 µl of a BC conidial suspension (5 x 10^5 conidia/ml) were inoculated.

For each treatment (non-treated, bacterial strain only, BC only, bacterial strain and BC) 10 berries were put on a sterile ceramic tray in a sterile glass chamber, containing a wet piece of sterile filter paper to maintain relative humidity of 95% inside the chamber, and incubated at 20 °C in the dark. Each treatment was carried out in triplicates.

Fruits were visually evaluated to determine fungal colonization of the berries at 5 days after the inoculation. The results were expressed as visual classes as according to the scale presented in a previous work (Vercesi *et al.* 2014). Visual classes were transformed in a percentage colonization index (C%I) according to the formula proposed by Townsend and Heuberger (1943). The values of C%I were compared between treatments performing one-way ANOVA, followed by Tukey's exact post-hoc test ($p < 0.05$).

3.2.2.2 Assays on grapevine leaves

For this assay, leaves of *V. vinifera* var. Red Globe were used, as described in Materials. Each leaf was washed twice in sterile distilled water and subsequently dipped for 12 seconds in either a solution of bacterial cells of each strain (10^5 CFU/ml, in Ringer's solution) or in sterile Ringer's solution (non-treated control).

After the dipping, 3 leaves for each treatment were placed in Petri dishes containing Water-Agar medium, for a total of 5 plates for each treatment (15 leaves each).

The BC inoculum was a 0.5 cm in diameter mycelial plug excised with a sterilized cork borer from the edges of an actively growing fungal culture placed in the middle of each leaf.

After the BC inoculum, each plate was sealed with parafilm and incubated in the dark at 20 °C for 7 days.

Severity of symptom was evaluated visually as reported above, measured at 3, 5, and 7 dpi, and converted to C%I.

The values of C%I were compared between treatments performing one-way ANOVA, followed by Tukey's exact post-hoc test ($p < 0.05$).

3.2.3 *In planta* assays

All assays carried out *in planta* to test the ability of the 7 bacterial strains to promote growth of a plant host and to exert a biocontrol effect against different pathogens will be described in this chapter.

3.2.3.1 Pathogenicity tests and colonization assays

The first step in determining the effect that the candidate BCA could have on a plant host was to determine whether they could colonize a host and that they did not develop symptoms on the host. Pathogenicity tests were carried out using all seven bacteria, both by inoculating the bacteria at the root by dipping the root in a solution of bacterial cells (10^5 CFU/ml) in Quadrato d'Asti pepper plants, and by leaf infiltration of such a solution (approximately 200 μL per infiltration) of *N. benthamiana* and *N. tabacum*.

Likewise, the effective ability of the selected pathogens to develop symptoms was tested for *P. syringae* strain DC3000 and DC3000::*gfp*, as well as the virus CymRSV. *P. syringae* strains were inoculated by leaf infiltration (10^5 CFU/ml in Ringer's solution, approximately 200 μL per infiltration) in *N. benthamiana* and *N. tabacum*. CymRSV was inoculated mechanically using infected leaves ground in Norit buffer (1:10 w/w concentration) as inoculum. Development of symptoms (necrotic areas, yellowings, crinkling, stunted growth, wilting) was checked over the course of two weeks.

Successful endophytic colonization could be assayed only for strains 255-7:*rfp*, 260-02:*gfp*, and R8::*gfp*, as they had a molecular marker that allowed both tracking and selective isolation. All three strains were inoculated in Zebo pepper plants by root dipping and, after one month, leaves were collected from the plants and used to (i) extract total nucleic acids, which were then used to amplify the gene encoding for the RFP or GFP protein, as appropriate, using a specific PCR (see Appendix) and they were (ii) ground in Ringer's solution, serially diluted to 10^{-3} of the initial

concentration. Each dilution was plated on 3 LBA plates containing kanamycin to recover the transformed bacterial strain inoculated in the plant. The successful colonization was also checked in the plants used for plant-growth promotion assays (see 3.2.3.2), following this same procedure on plants one month after the inoculation of the bacteria.

Furthermore, for strain 260-02:*gfp* and DC3000::*gfp*, epiphytic colonization was confirmed directly through confocal fluorescence microscopy (Olympus FluoView FV1000), determining the presence of the strain on the surface of Zebo roots at 3 and 6 days post inoculation. Whole roots cleaned from soil were excised from the plant with a scalpel, directly put on a glass slide and covered with a few drops of sterile Ringer's solution, with no further preparation, and were examined at the microscope. Absence of fluorescence similar to GFP in non-treated samples was evaluated as well to exclude the possibility of false positives. This microscopy analysis was carried out in collaboration with Dr. Stéphane Compant, PhD, at the Austrian Institute of Technology (AIT), Bioresources unit.

3.2.3.2 Plant-growth promotion ability

The ability of the seven strains to promote the growth of a plant host was tested on different plant species, as reported in **Table 2**. More possible plant hosts were assayed for the marked strains 255-7:*rfp*, 260-02:*gfp*, and R8::*gfp*, while only pepper Zebo was used for the other strains, having shown a quite good response to the inoculation with these first three strains.

In each assay, 2 weeks old seedlings were inoculated by root dipping: plants were carefully uprooted, avoiding damage to the roots, most of the soil clinging to the roots was washed away with tap water, and the roots were dipped for 30 minutes in a suspension of bacterial cells (10^5 CFU/ml in Ringer's solution) or in Ringer's solution for the negative control. After the dipping, the plants were put in 10 cm in diameter pots containing potting soil and kept in greenhouse at a temperature between 25 °C and 28 °C. Each treatment was carried out on 7 plants to obtain biological replicates. For the trials carried out during spring and summer (A), no artificial lights were used, as natural sunlight entering in the greenhouse was sufficient for growth. In the trials carried out in autumn and winter (B and C), artificial lights were used to provide the plants with 14 hours of light per day.

In all assays, the height of plants was measured weekly over a period of five weeks and, before comparing between the treatments, the height of each plant at the time of inoculation was subtracted from the following measures, in order to normalize the starting height parameter. Therefore, the height of plants will be expressed as Δh, calculated as $h_i - h_0$, where h_i is the height measured at any given week and h_0 is the initial height of the plant.

At the end of the five weeks of growth, the fresh weight of the plants was measured as well, to see if the bacteria had an effect on production of biomass, and not merely plant height.

Since the plants grown in different trials cannot be compared directly, the data for comparing the effect between bacteria utilized in different trials will be presented as percentage of height difference (%h), calculated as $(\Delta h_t/\Delta h_c)*100$, where Δh_t is the average Δh of any treatment, and Δh_c is the average Δh of the non-treated control plants. Applying this formula, the non-treated control plants have a fixed value of 100, and any value above 100 expresses an increase in growth, while any value below 100 expresses a reduction in growth, regardless of the actual height in centimeters of the analyzed plants.

These parameters were compared between the different treatments in the same trial using an ANOVA, optimized for repeated measures in the case of Δh and %h, followed by Tukey's exact post-hoc test ($p < 0.05$).

Table 2 Plant-growth promotion assays carried out in the present study for each of the examined strains. All plants used in the study are reported as rows, while the strains are reported in columns. An X indicates that the combination of strain and host has been assayed. Letters from A to C indicate the three different assays carried out using *C. annuum* Zebo F1 as plant host.

Plant material	255-7	255-8	260-02	S4C11	R1	R8	R16
Quadrato d'Asti	X					X	
Zebo F1	X_C	X_B	X_A	X_C	X_C	X_B	X_B
Tasty Green	X					X	
Genovese	X					X	
N. benthamiana	X					X	
N. tabacum	X					X	
Nano Bobis	X					X	
Rio Grande			X				
San Marzano 2	X					X	
Sibari F1			X				
Beatrice F1			X				
Violetta lunga F1	X					X	

3.2.3.3 Evaluation of methylation level in Zebo F1 pepper plants

An additional assay was carried out on the Zebo plants used in trial C: after 20 days from the bacterial inoculation, 1 gram of leaf tissue was sampled from each treated and non-treated control plant (7 plants per treatment) and total nucleic acids were extracted from these samples (see Appendix). Percentage of cytosine methylated in position 5 (5-mC) in these samples was evaluated with the use of 5-mC DNA ELISA kit (Zymo Research), following the producer's instructions. Measurements of absorbance at 450 nm for each sample and each point of the standard curve (0%, 10%, 25%, 50%, 75%, and 100% of C-methylated DNA) were performed in triplicate. Linear regression of the values obtained by the standards allowed to determine the percentage of 5-mC present in the samples. These values were then compared among the treatments by performing one-way ANOVA, followed by Tukey's exact post-hoc test ($p < 0.05$)

3.2.3.4 *In planta* biocontrol against *R. solani*

Biocontrol assays against the fungal pathogen RS were carried out in parallel to plant-growth promotion assays on Zebo plants. These assays were carried out on 7 plants per treatment.

The fungal inoculum used in this assay was obtained by air drying an active culture of RS, incubated on pearl millet at 30 °C for 3 weeks.

The fungal inoculum was added to the potting soil, previously sterilized by autoclaving 4 times at intervals of approximately 16 hours between each sterilization, at a final concentration of 20 g/kg of soil, one week before the plants were inserted in the pots.

The plants were visually examined for the presence of symptoms typical of *R. solani* (post-germination damping off, root rot) for the period of 5 weeks and, at the end of the experiment, the roots were examined as well. In particular, the presence of stunted growth was considered the main symptom, so for this pathogen the considered parameters were Δh and fresh weight. These parameters were compared between non-treated plants (both inoculated with RS and healthy) and plants that were inoculated with the bacterial strain and RS by performing one-way ANOVA, followed by Tukey's exact test ($p < 0.05$)

Roots samples (1 gram) were collected from both RS-inoculated plants and non-treated controls. Total nucleic acids were extracted from these samples and were used in a PCR assay with specific primers (ST-RS1, ITS4) to determine the presence of RS (see Appendix).

3.2.3.5 *In planta* biocontrol against *Pseudomonas syringae* pv. tomato DC3000

Biocontrol against the bacterial pathogen *P. syringae* pv. tomato DC3000 were carried out in parallel to plant-growth promotion assays on Zebo plants. These assays were carried out on 7 plants per treatment.

DC3000 was incubated overnight in LB medium at 24 °C with shaking (150 rpm) to allow growth. Then the cells were pelleted by centrifugation (5000x g, 5 minutes) and resuspended in Ringer's solution to a concentration of 10^5 CFU/ml. This suspension was sprayed on the leaves of Zebo plants (approximately 15 ml per plant) 2 weeks after they were treated by root dipping.

The plants were visually examined for the presence of symptoms typical of *P. syringae*, namely necrotic spots, for the following 2 weeks for all three trials.

On each examination, the number of necrotic spots per plant was recorded and compared between the treatments and non-treated control by Mann-Whitney non-parametric test.

For strain 260-02 only, an additional assay was carried out, on both Zebo and Sibari plants, in an attempt to visually determine its effect in preventing damage caused by strain DC3000. In this experiment, one week old plants that were either inoculated by root dipping with strain 260-02, strain DC3000, or mock-treated with Ringer's solution, had their leaves sprayed with DC3000::*gfp* strain one week after the root dipping, and the colonization pattern of this pathogen on the leaves was examined at 3 and 6 dpi. This microscopy analysis was carried out in collaboration with Dr. Stéphane Compant, PhD, at the AIT, Bioresources unit.

3.2.3.6 *In planta* biocontrol against Cymbidium Ringspot Virus

Biocontrol against the viral pathogen CymRSV was carried out in parallel to plant-growth promotion assays on Zebo plants. These assays were carried out on 7 plants per treatment.

CymRSV was inoculated on *N. benthamiana* plants two weeks prior to the inoculation on Zebo plants and, when the plants displayed intense symptoms, leaves were harvested, ground in a mortar

with addition of Norit-buffer (1:10 w/w), and spread on the surface of Zebo leaves, sprayed with carborundum powder to abrade the surface and help entry of the pathogen.

This operation was carried out two weeks after the root dip treatment.

Symptoms of the virus were evaluated as percentage of systemic infected leaves (SIL%) during trial A, calculated as SIL/NL * 100, where SIL is the number of leaves that show symptoms from systemic infection, and NL is the number of leaves that were not directly inoculated with the virus.

During trials B and C, it was instead evaluated using a class system with three values: 0 means no symptom, 1 means symptoms developing locally on inoculated leaves, 2 means symptoms developing systemically on non-inoculated leaves. These classes were then converted in a percentage of virus infection (VI%) using the formula presented by Townsend and Heuberger (1943).

The data obtained were compared, between each treatment and the non-treated control, using the Mann-Whiteny non-parametric test.

Furthermore, the response of the inoculated plants was evaluated by semi-quantitative retro-transcription real-time PCR assays (sqPCR) amplifying the cDNA obtained by retro-transcribing the RNA extracted from plants of different treatments to compare the expression of two genes related to pathogen response and systemic resistance: *pr1*, *pal* (cit.). Also, the presence of the pathogen was quantified using specific primers for CymRSV (1738 L, 1933 R), designed during this study and already reported as part of a master thesis (Gennari, 2016-2017). All genes were normalized using the actin housekeeping gene for determining relative quantification using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All the primers and protocols used for these analyses are reported in the Appendix. These sqPCR analyses were carried out twice, once on samples collected the day after the virus inoculation, and once on samples collected 14 days after the virus inoculation.

3.2.4 *In silico* analysis of examined strains

All analyses carried out *in silico* on the genomes of the 7 bacterial strains will be described in this chapter.

3.2.4.1 Genome sequencing through Illumina HiSeq1000 and assembly

Genomic DNA was extracted from each endophytic strain. The strains were cultivated in LB medium at 24 °C overnight and the genomic DNA was extracted using GenElute Bacterial Genomic DNA Kit (Sigma-Aldich), following the manufacturer's instruction, with adjustments for Gram-positive bacteria in the case of strains S4C11, R1, and R16. Genomic DNA was quantified with the Qubit dsDNA HS Assay kit (Life Technologies), DNA purity and integrity were assessed at the Nanodrop 1000 spectrophotometer (Thermo Scientific) and by agarose gel electrophoresis, respectively.

Illumina libraries were produced starting from 1 µg of genomic DNA, which was sheared using the Covaris S220 instrument (Covaris Inc. Woburn, MA). Selection of DNA fragments of 500bp in length was conducted on 1.8% agarose gel and sequencing libraries were produced using the TruSeq DNA Sample Prep Kit (Illumina, San Diego, CA) following the manufacturer's instructions. Sequencing was performed on a HiSeq1000 instrument with 100-nucleotide paired-end protocol using the TruSeq PE Cluster v3 kit (Illumina, San Diego, CA) according to manufacturer's instructions.

Illumina reads underwent quality filtering and trimming using Sickle and were quality corrected with BayesHammer before being assembled de novo using the SPAdes 2.9.0 software, using multiple k-mer combinations in the range between 75 and 97.

These sequencing and assemblies were carried out in collaboration with the University of Verona, department of Biotechnologies.

3.2.4.2 Genome sequencing of strain R16 with hybrid Illumina-minION approach and assembly

For strain R16, a second genome sequencing experiment was carried out combining the data obtained through Illumina sequencing, described in the previous paragraph, and reads obtained from an Oxford Nanopore Technologies (ONT) minION technique.

The same genomic DNA utilized for the preparation of Illumina libraries was used also for the preparation of minION libraries.

DNA libraries for Oxford Nanopore sequencing were produced starting from 1.8 μg DNA that was randomly sheared to an average length of ~15Kbps using g-TUBEs (Covaris) centrifuged at 5000 rpm (Centrifuge 5424, Eppendorf) for 60 seconds. The size of DNA fragments was assessed using the Genomic DNA Screen Tape at the TapeStation 2200 (Agilent Technologies). The library preparation was performed using components from the Genomic DNA Sequencing Kit SQK-MAP006 (Oxford Nanopore Technologies) and following Version R9 of the Oxford Nanopore protocol MAP006. DNA nicks were repaired using the FFPE DNA repair mix (New England Biolabs, NEB) and End-repair and dA-tailing were performed using the Ultra II End Prep Module (New Englands Biolabs, NEB Ipswich, USA) according to manufacturer's instructions. The utilized adapter consisted of a linear double strand sequence and a hairpin sequence that links the positive and negative strand of each fragment to allow the sequencing of both strands (2D reads). The prepared library was quantified using a Qubit (Life Technologies) to estimate the total amount of DNA prior to loading the MinION and prepared for loading as indicated by Oxford Nanopore Technologies.

Prior to sequencing the MinION flowcell quality control was carried out in order to determine the number of available pores for the sequencing. Before the library loading, the MinION Flow Cell was primed according to the manufacturer's instructions. The library mix was loaded into the MinION Flow Cell and the sequencing was performed using the "MAP_48Hr_Sequencing_Run_SQK_MAP006" protocol on the MinKNOW software.

The obtained ONT reads were utilized, in conjunction with Illumina reads, for another de novo assembly carried out in SPAdes 2.9.0 in hybrid mode with the nanopore option.

This sequencing and assembly were carried out in collaboration with the University of Verona, department of Biotechnologies.

3.2.4.3 Genome annotation and functional analysis

Assembled genomic sequences were searched for putative assembled plasmid genomes by BLAST search against NCBI plasmid genomes database. Gene annotation was performed using RAST web service and functional annotation of protein coding genes was improved with Blast2GO software (ver. 2.8). Production of secondary metabolites was predicted using the antiSMASH 3.0 online tool.

Furthermore, functional analysis of the genomes was manually carried out, searching for genes known for being associated to endophytic lifestyle, plant colonization capabilities, as well as being associated to beneficial plant bacteria.

The annotations were carried out in collaboration with the University of Verona, department of Biotechnologies.

3.2.4.4 Comparative pangenome analysis

A comparative analysis at pangenome level was carried out for strain 260-02, searching for differences between its genome and that of other strains of *P. syringae*, which are known pathogens.

For this analysis, the genomes of a list of *P. syringae* strains, downloaded from NCBI (**Table 3**), and the genome of strain 260-02 were annotated with the PROKKA software.

After this step, performed to have a homogeneous annotation of the genomes, they were compared using the Roary software. The main output of the software was a gene presence/absence table, which was manually examined to search for features that could be related to the different phenotype of the bacterial strains.

The pangenome analysis was carried out in collaboration with the AIT, Bioresources unit.

Table 3 Database of the genomes utilized for the comparative pangenome analysis. In columns are reported the names of the strains, their pathovar, and their accession number. * Strain 1448A is from *P. savastanoi*, rather than *P. syringae* but was included as, until recently, the strain and whole pathovar *phaseolicola* was considered to be part of *P. syringae*.

Strain	Pathovar	AN
1448A	Phaseolicola*	NC_005773.3
ATCC_10859	Lapsa	NZ_CP013183.1
B301D	Syringae	NZ_CP005969.1
B728a	Syringae	NC_007005.1
CC1557	N/A	NZ_CP007014.1
DC3000	Tomato	NC_004578.1
HS191	Syringae	NZ_CP006256.1
ICMP 18708	Actinidiae	NZ_CP012179.1
ICMP 18884	Actinidiae	NZ_CP011972.2
UMAF 0158	Syringae	NZ_CP005970.1

3.3 Appendix

In this chapter, all media, buffers, and protocols used transversally through different experiments or that are for routine laboratory practices will be reported.

3.3.1 Culture media

For the different protocols utilized in this work, several bacterial culture media, both solid and liquid, were utilized. All media were sterilized before use by autoclaving at 121 °C for 15 minutes. The media used in the present work were prepared as follows:

- Luria-Bertani Broth (LB): Tryptone 10 g/l, yeast extract 5 g/l, sodium chloride 10 g/l (Luria and Burrows, 1957)
- Luria-Bertani Agar (LBA): prepared as LB, but with the addition of 15 g/l of agar
- Potato Dextrose Agar (PDA): Potato starch 4 g/L, dextrose 20 g/L, Agar 15 g/L. Prepared from dehydrated culture medium bought from Difco™
- Tryptone-glucose-yeast (TGY): Tryptone 5 g/l, yeast extract 3 g/l, sodium chloride 5 g/l, glucose 1 g/l
- TGY 2x: prepared as TGY, but with double concentration of each component for each liter of water
- Tryptone-glucose-yeast Agar (TGYA): prepared as TGY, but with the addition of 15 g/l of agar
- TGYA 2x: prepared as TGYA, but with double concentration of each component for liter of water
- Water-Agar: 15 g/l Agar for liter of water

Additionally, when used with strains carrying a selective marker for antibiotic resistance, each of the medium mentioned above could be added with 50 μg/ml of kanamycin, obtained by adding the opportune quantity of a stock solution of kanamycin sulfate (Sigma Aldritch) in sterile water (50 mg/ml).

3.3.2 Buffers and solutions

The following buffers were used in the present work:

- CTAB 2.5%: prepared by adding 100mM Tris pH 8, 1,4 M NaCl, 50mM ETDA pH 8; 1% w/v PVP, 2.5 % w/v CTAB in 1 liter of water
- Norit-buffer: sodium phosphate and potassium phosphate 0.05 M pH 7, diethylthiocarbamic acid (DIECA) 5 mM, EDTA 1 mM
- Ringer's solution: prepared by dissolving pre-made tablets from [ditta] in an appropriate volume of distilled water (1 tablet for each 500 ml of water)
- Sterlizing solution: containing 80 ml of NaOCl (7%), 100 ml of ethanol (96%) and 820 ml of sterile distilled water.
- Tris-Borate-EDTA (TBE): Trizma base (162 g/l), orthoboric acid (46.3 g/l), ethyldiaminotetraacetic acid (EDTA) (9.5 g/l)

3.3.3 Preparation of liquid culture for bacterial inoculi

Liquid culture used to prepare bacteria inoculi was obtained as follows: 1 colony of the strain was picked from a plate and added to 3 ml of LB broth in a 15 ml tube, and incubated at 25 °C overnight with constant shaking at 180 rpm. The following day, 1 ml from this culture was taken and added to 100 ml of LB broth in a 500 ml conical flask and incubated at 25 °C with constant shaking until the desired bacterial growth was obtained. The concentration of bacteria in the medium was assayed by spectrophotometry at a wavelength of 600 nm. The bacterial cells were then pelleted by centrifugation at 8000 xg for 10 minutes, resuspended in sterile Ringer's solution and diluted to the desired concentration.

3.3.4 Total nucleic acid extraction from plant material

Whenever DNA was extracted from plant material, the following protocol, following the one proposed by Doyle and Doyle (1990) with some modifications, was used:

- Weigh 1 g of sample in a Bioreba extraction bag, add 5 ml of CTAB 2.5 % buffer, pre-heated at 65 °c and added with 0.5% w/v ascorbic acid
- Grind the content of the bag with a mechanical pestle until it becomes a homogeneous solution
- Take 800 µL of sample from each bag and transfer it to a 2 mL tube
- Incubate at 65 °C for 30 minutes, stirring the content of the tubes by inversion every 5 minutes
- Add 1 volume of chloroform:isoamylic acid (24:1) and vortex until the two phases unite
- Centrifuge at 13000 rpm on a bench centrifuge for 15 minutes
- Pipette only the idrophylic part (supernatant, approximately 600 µL) and place it in a new 1.5 mL tube
- Precipitate the nucleic acids by adding 2/3 volumes of cold isopropanol, mix by inversion, and store at -20 °C for at least 10 minutes
- Centrifuge at 13000 rpm on a bench centrifuge for 10 minutes, and discard the supernatant
- Wash the pellet with 800 µL of 70% ethanol for 5 minutes before centrifuging (13000 rpm, 5 minutes) and discarding the supernatant
- Completely dry the pellet before adding 400 µL TE buffer and incubate it at room temperature for 15 minutes
- Precipitate the nucleic acids by adding 40 µL of NaOAc 3 M (pH 5.2) and 800 µL of cold pure ethanol and mixing by inversion
- Centrifuge at 14000 rpm on a bench centrifuge, discard the supernatant
- Wash the pellet twice with 800 µL of ethanol incubating for 10 minutes at room temperature before centrifuging (13000 rpm 5 minutes)
- Completely dry the pellet resuspending it in 100 µL of TE buffer and storing the nucleic acids at -20 °C

3.3.5 Extraction of RNA from plant material

In order to evaluate gene expression in inoculated Zebo plants, total RNA was extracted from the leaves of the plants, following the method described by Gambino *et al.* (2008) with some modifications:

- Weigh 0.5 g of leaf in a Bioreba extraction bag, add 5 mL of CTAB 2.5% buffer, pre-heated at 65 °C and added with 1% w/v sodium metabisulfite
- Grind the content of the bag with a mechanical pestle until it becomes a homogeneous solution
- Take 850 µL of sample from each bag and transfer it to a 2 mL tube
- Incubate at 65 °C for 30 minutes, stirring the content of the tubes by inversion every 2-3 minutes
- Add 1 volume of chloroform:isoamylic acid (24:1) and vortex until the two phases unite
- Centrifuge at 10000 rpm on a bench centrifuge for 10 minutes at 4 °C
- Pipette only the idrophylic part (supernatant, approximately 600 µL) and place it in a new 1.5 mL tube
- Add 0.5 volumes of lithium chloride 9 M and incubate for a maximum of 30 minutes on ice
- Centrifuge at 14000 rpm on a bench centrifuge for 20 minutes at 4 °C
- Discard the supernatant and resuspend the pellet in 450 µL of SSTE buffer, pre-heated to 65 °C
- Repeat the chloroform:isoamylic alcohol extraction step
- Add 0.7 volumes of isopropanol and 0.1 volumes of NaOAc 3 M (pH 5.2) to the collected supernatant and mix by inversion
- Centrifuge at 14000 rpm on a bench centrifuge for 20 minutes at 4 °C
- Discard the supernatant, wash the pellet with 500 µL of cold 70% ethanol before centrifuging (14000 rpm, 5 minutes, 4 °C)
- Completely dry the pellet before resuspending it in sterile DEPC water
- Store at -80 °C

3.3.6 PCR conditions

The PCR reactions carried out as part of the study were carried out with the conditions described below.

Each PCR reaction was performed in a volume of 25 µL and contained: 1x Buffer, $MgCl_2$ 3 mM, dNTPs 0.2 mM each, primers 0.4 mM each, Taq polymerase 1.25 U, 1 µL of template DNA, and water to reach the volume of 25 µL.

The thermal cycle constituted of an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, and elongation at 72 °C for 1 minute, ending with a longer final elongation step of 10 minutes at 72 °C.

All PCR products obtained were visualized by 1% agarose gel electrophoresis in TBE buffer, stained with Midori Green dye.

The primer pairs used in this study are reported in **Table 4**.

3.3.7 Retrotranscription

In order to evaluate gene expression, RNA was retrotranscribed to cDNA using the following reaction:

- Digest all residual DNA by adding 1 µL of DNase enzyme and 1 µL of DNase buffer 10x to 8 µL of RNA, and incubate at 37 °C for 30 minutes
- Add 1 µL of EDTA 25 mM and incubate at 65 °C for 10 minutes
- Proceed to retrotranscribe the RNA by adding 1 µL of dNTPs (10 mM), 0,5 µL of random hexamers (0.2 µg/ µL), and 0.5 µL of water, incubate at 90 °C for 5 minutes and then instantly cool to 4 °C by putting the tubes in ice
- Add 2 µL of DTT (0.1 M), 2 µL of retrotranscription buffer 10x, 2 µL of $MgCl_2$ (50 µM) and 1 µL of M-MLV retrotranscriptase; incubate at 25 °C for 10 minutes, 37 °C for 45 minutes and 70 °C for 15 minutes
- Dilute the obtained cDNA with one volume of water and store at -20 °C

3.3.8 Semi-quantitative real-time PCR conditions

All real-time assays performed in this study were carried out using a two-steps SYBR® Green system in a StepOnePlus™ thermocycler (Applied Biosystems™).

Each reaction was carried out in a 10 µL volume, containing: 2 µL of cDNA, 400 µM of each primer, 1x Power SYBR® Green PCR Master Mix, and water to reach the volume of 10 µL.

For each gene examined, each sample was amplified in triplicates to obtain a more precise value for the threshold cycle.

The primers utilized in these real-time PCR assays are reported in **Table 4**.

Table 4 List of all primers used in this study, for PCR and sq-PCR assays. The first column reports the name of the primer, the second the type of assay it was used for, the third the sequence of each primer, and the fourth the reference from which the primer was obtained.

Primer	Assay	Sequence	Reference
fGFP	PCR	CATATGAAACAGCATGACTTTT	Valtorta, 2012-2013
rGFP		AACTCAAGAAGGACCATGTGGT	
fRFP	PCR	ACCCATATGAGGTCTTCCAAGAAT	Valtorta, 2012-2013
rRFP		GGCGGATCCCTAAAGGAACA	
ST-RS1	PCR	AGTGTTATGCTTGGTTCCACT	Lievens *et al.*, 2006
ITS4		TCCTCCGCTTATTATTGATATGC	
PR-1a F	sq-PCR	TCAAGTAGTCTGGCGCAACTC	Wang *et al.*, 2009
PR-1a R		CCAGTTGCCTACAGGATCGTA	
PAL F	sq-PCR	CTTTGATGCAGAAGCTGAGACA	Wang *et al.*, 2009
PAL R		TCGTCCTCGAAAGCTACAATCT	
ß-act F	sq-PCR	ACATTGTGCTCAGTGGTGGTACT	Wang *et al.*, 2009
ß-act R		CCACCTTAATCTTCATGCTGCT	
1738 L	sq-PCR	CATTGGTCTCGACGCGTCTA	Gennari, 2015-2016
1933 R		ACTCATACGACAGCCCTCCT	

4 Results

4.1 Results of *in vitro* assays

This chapter reports the results obtained from all the *in vitro* biocontrol assays carried out in *in vtiro* conditions against fungal phytopathogens.

4.1.1 Dual-culture assays

Dual-culture assays allowed to determine the ability of the 7 endophytic bacterial strain to control, in *in vitro* conditions, the growth of 5 different phytopathogenic fungi: *Aspergillus* sez. *nigri* (AN), *Botrytis cinerea* (BC), *Fusarium verticillioides* (FV), *Phomopsis viticola* (PV), and *Rhizoctonia solani* (RS). As the most significant results were obtained at 14 days of co-culture, data obtained from 5 and 7 days of co-culture won't be reported. As a general observation, when a strain was capable of inhibiting fungal growth, the effect was registered already at 5 dpi and became higher after 14 days of co-culture.

4.1.1.1 Inhibition of *Aspergillus* sez. *nigri*

All 7 bacterial strains managed to reduce the growth of AN during dual-culture assays, with an effect that ranged between less than 50% of growth reduction (strain 255-7 GIP = 46.8) to more than 90% of growth reduction (strain R8 GIP = 94.7), with 5 out of 7 strains showing a GIP value close to 90%, and only 2 of them showing 60% or lower (**Figure 3A**).

In particular, in the control plates of AN the measurement of the growth was made difficult by the presence of several secondary colonies originated from spores, which this fungus produces in great amount, and the production of these spores is easily seen by the black dots covering the growing colonies (**Figure 3B**). In treated plates, the growth of the fungus is instead limited to almost transparent mycelium, without the production of spores, and the presence of secondary colonies is not detected, as shown in **Figure 3C**, using as example the control obtained in dual-culture with strain 260-02.

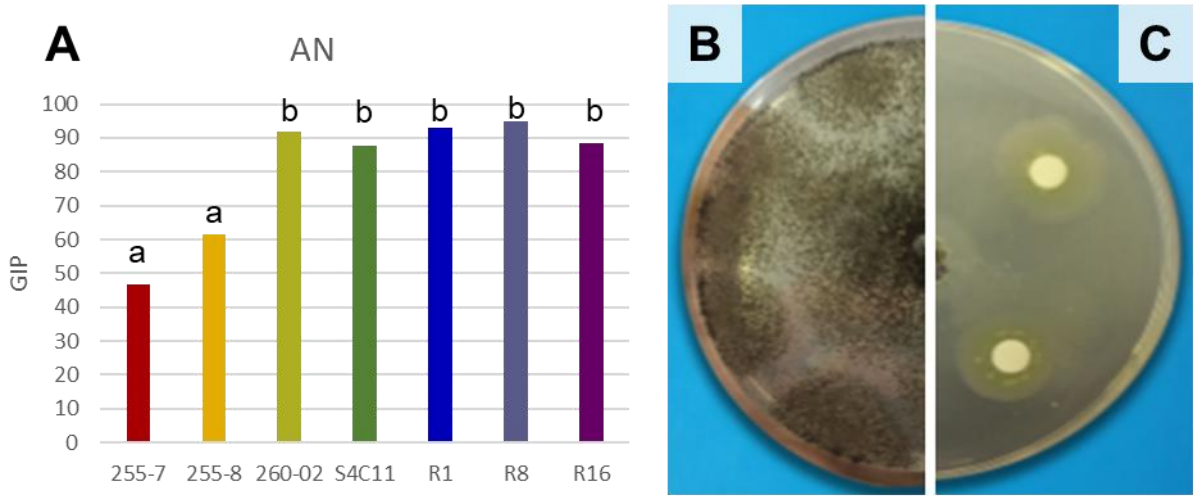


Figure 3 Results obtained from dual-culture assays regarding *Aspergillus* sez. *nigri* (AN) after 14 days of co-culture. **A)** graph reporting the GIP (growth inhibition percentage) with the different bacterial strains. Different letters indicate statistically significantly different results according to one-way ANOVA, followed by Tukey's exact test post-hoc ($p < 0.05$); **B)** picture of a non-treated control plate, showing the growth of the fungus and production of spores on TGYA medium; **C)** example of a treated plate, containing strain 260-02, showing effect of dual-culture on AN: greatly reduced mycelial growth and no production of spores is visible.

4.1.1.2 Inhibition of *Botrytis cinerea*

All 7 bacterial strains managed to reduce the growth of BC during dual-culture assays, with an effect that ranged between about 60% (strain 260-02 GIP = 64.5) to a complete inhibition of growth (e.g. strain R16 GIP = 100). Only three strains (255-8, R1, and R16) managed to achieve this complete inhibition of growth, but the others still showed a high percentage of inhibition (**Figure 4A**).

In particular, in the control plates the growth of the fungus was easily visible by the presence of brown-yellow mycelium growing on the TGYA and, after about one week from the inoculation, the development of a darker coloration as spores were produced (**Figure 4B**). In treated plates, the fungus could either not develop at all, leaving only a very faint halo surrounding the mycelial plug (**Figure 4C**), or developed only a smaller, but highly compact, mycelium, accompanied by the formation of a dark brown coloration in the growing substrate (**Figure 4D**).

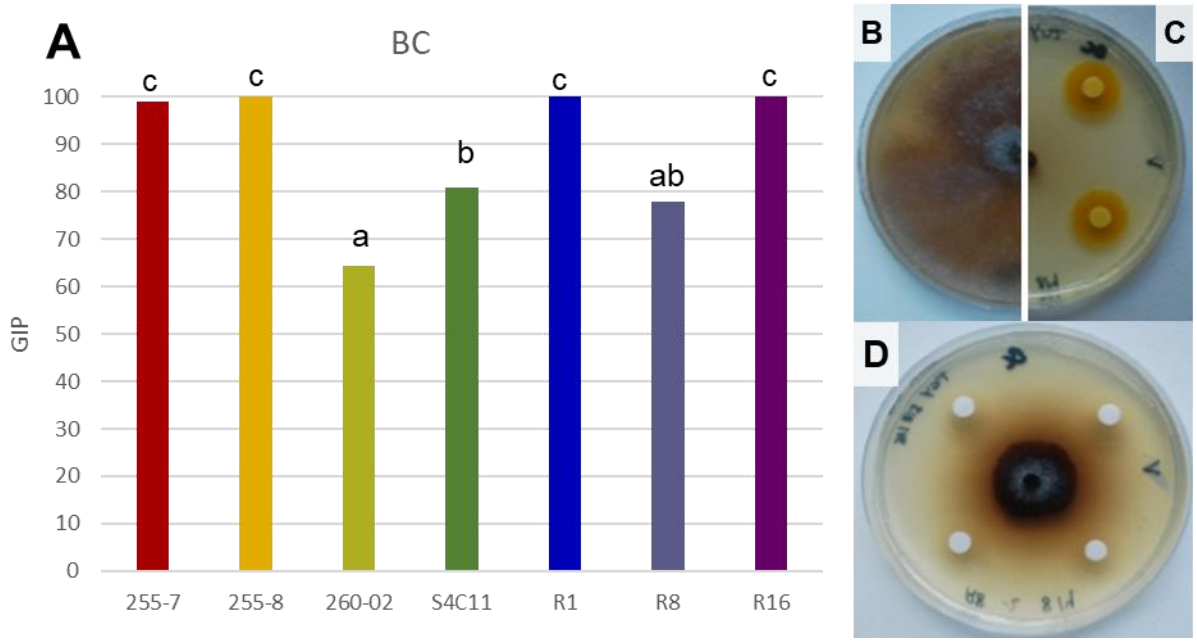


Figure 4 Results obtained from dual-culture assays regarding *Botrytis cinerea* (BC) after 14 days of co-culture. **A)** graph reporting the GIP obtained with the different bacterial strains. Different letters indicate statistically significantly different results according to one-way ANOVA, followed by Tukey's exact test post-hoc ($p < 0.05$); **B)** picture of a non-treated control plate, showing the growth of the fungus and production of spores on TGYA medium; **C)** example of a treated plate, containing strain R16, showing effect of dual-culture on BC: greatly reduced mycelial growth and no production of spores is visible; **D)** example of a treated plate, containing strain R8, showing effect of dual-culture on BC: compact, dark-colored mycelium and a brown halo spreading in the medium.

4.1.1.3 Inhibition of *Fusarium verticillioides*

None of the 7 bacterial strains managed to exert a satisfactory inhibition of the growth of FV. A minimal inhibition of growth was observed in the case of strain 260-02 (GIP = 7.0), while no inhibition of growth was seen in the dual-culture assay with the other 6 strains (**Figure 5A**).

Still, all the strains changed the morphology of the FV mycelium in the close proximity of the bacterial colonies: while in the non-treated plates the mycelium growth was homogeneous, showing a powdery white surface in the whole plate (**Figure 5B**), in the treated plates there was a noticeable change in the quality of the fungal colony, with mycelium becoming less compact and gaining a flaky appearance especially in the near proximity of bacterial colonies, but no actual growth inhibition (**Figure 5C**).

The presence of an effect on the mycelium of FV was also proven by optical microscopy performed on the plates treated with strain R16, in which it is possible to see a difference compared to the non-treated control: the hyphae having been degraded by the bacteria, possibly through a chitinase, and no spores being visible (**Figure 5E**), while normal hyphae and the large amount of spores normally surrounding them is shown in **Figure 5D**.

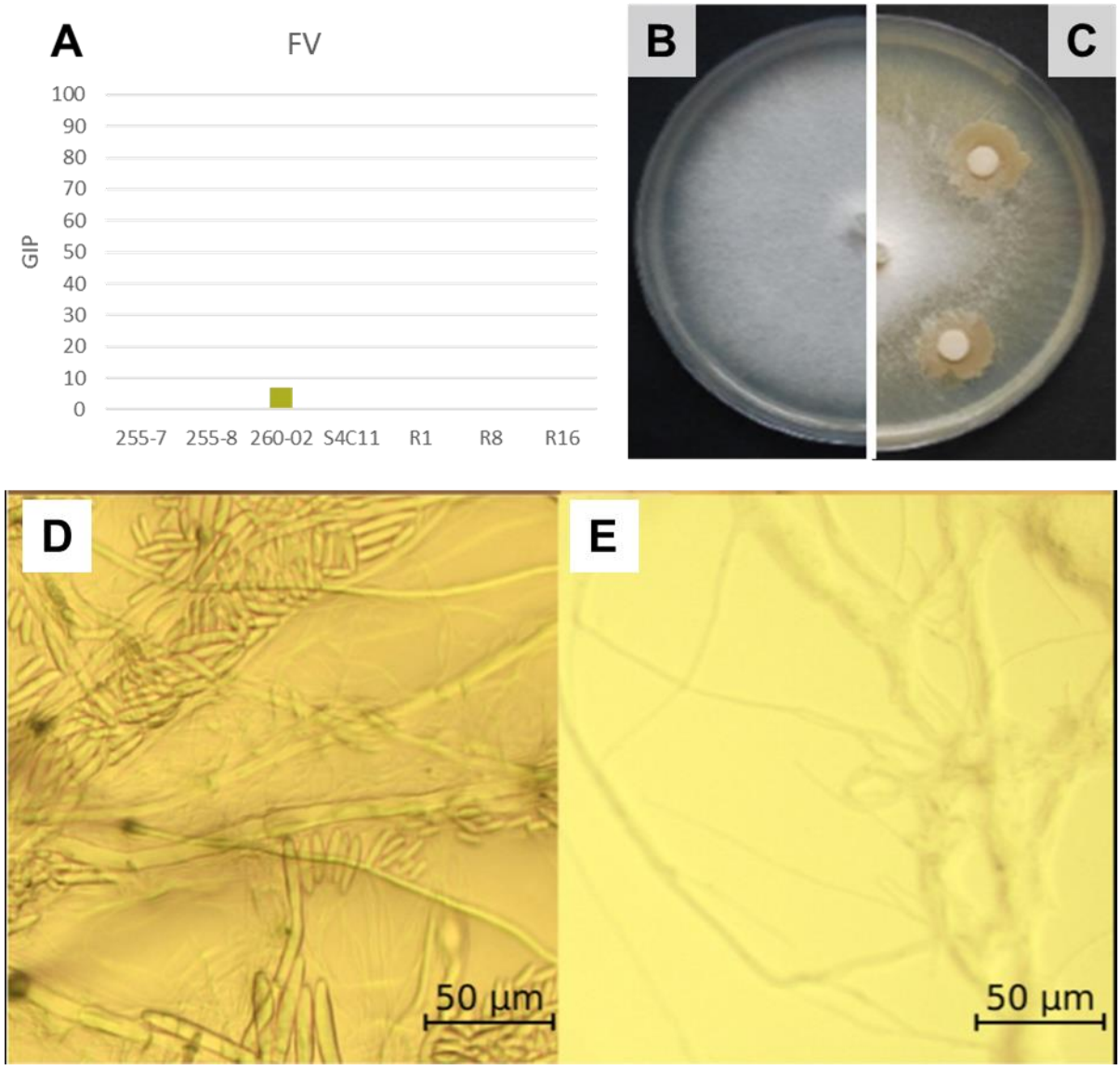


Figure 5 Results obtained from dual-culture assays regarding *Fusarium verticillioides* (FV) after 14 days of co-culture. **A)** graph reporting the GIP obtained with the different bacterial strains; **B)** picture of a non-treated control plate, showing the growth of the fungus and production of spores on TGYA medium; **C)** example of a treated plate, containing strain R16, showing effect of dual-culture on FV: modified structure of the mycelium and flaky appearance; **D)** optical microscopy of a non-treated plate, showing hyphae and spores of FV in normal conditions; **E)** optical microscopy of an R16-treated plate, showing the degenerated hyphae and lack of spores near the colonies.

4.1.1.4 Inhibition of *Phomopsis viticola*

All 7 bacterial strains managed to reduce the growth of PV during dual-culture assays, with an effect ranging between approximately 50% (strain R8 GIP = 47.0) and over 90% (strain 255-8 GIP = 93.0), with 6 out of 7 strains showing a moderate or low level of inhibition, lower than 70% (255-7, 260-02, S4C11, R1, R8, R16), and only 1 strain showing a high level of inhibition (255-8) (**Figure 6A**).

In particular, PV showed a slower growth in diameter compared to the other phytopathogenic fungi considered, and developed in a characteristic pattern of concentric rings, which usually did not reach the outer border of the Petri dish (**Figure 6B**). Also, in these experimental conditions, no development of spores was observed for PV. In treated plates, the inhibition was detectable as a simple reduced development of the diameter of the fungal colony, with no modifications to the morphology of the fungi (**Figure 6C**)

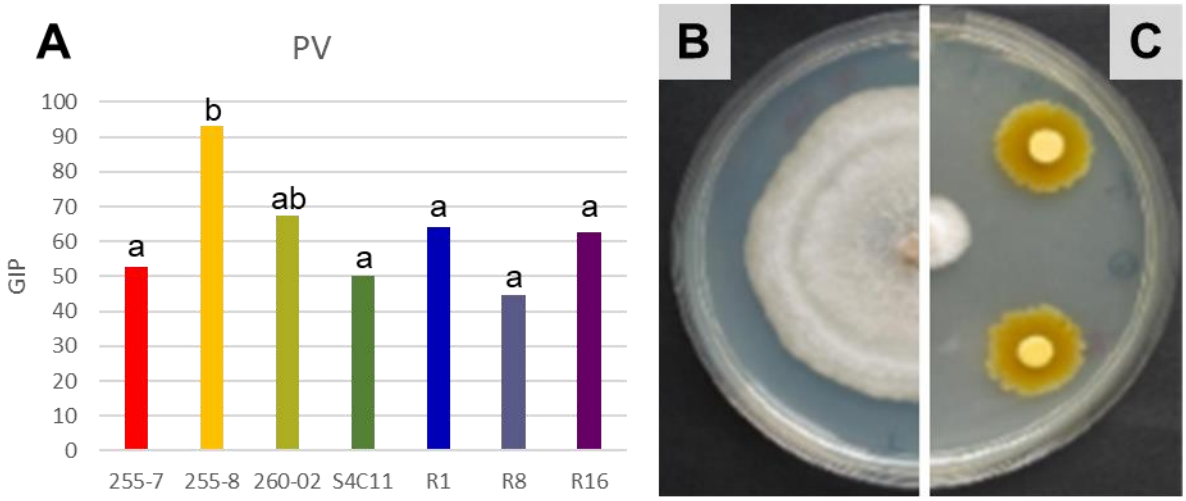


Figure 6 Results obtained from dual-culture assays regarding *Phomopsis viticola* (PV) after 14 days of co-culture. **A)** graph reporting the GIP obtained with the different bacterial strains. Different letters indicate statistically significantly different results according to one-way ANOVA, followed by Tukey's exact test post-hoc ($p < 0.05$); **B)** picture of a non-treated control plate, showing the growth of the fungus on TGYA medium; **C)** example of a treated plate, containing strain R16, showing effect of dual-culture on PV: greatly reduced mycelial growth;

4.1.1.5 Inhibition of *Rhizoctonia solani*

All 7 strains were able to reduce the mycelial growth of RS during dual-culture assay, with an effect ranging from around 80% (strain R1 GIP = 81.4) to complete inhibition of the growth (e.g. strain 260-02 GIP = 100), with all strains showing a strong suppressive effect on the growth of this fungus (Figure 7A).

In particular, RS grew to quickly fill the non-treated Petri dishes with a brown mycelium (**Figure 7B**), while in treated plates the growth was stopped close to the origin point, or completely negated by the presence of the bacteria (**Figure 7C**).

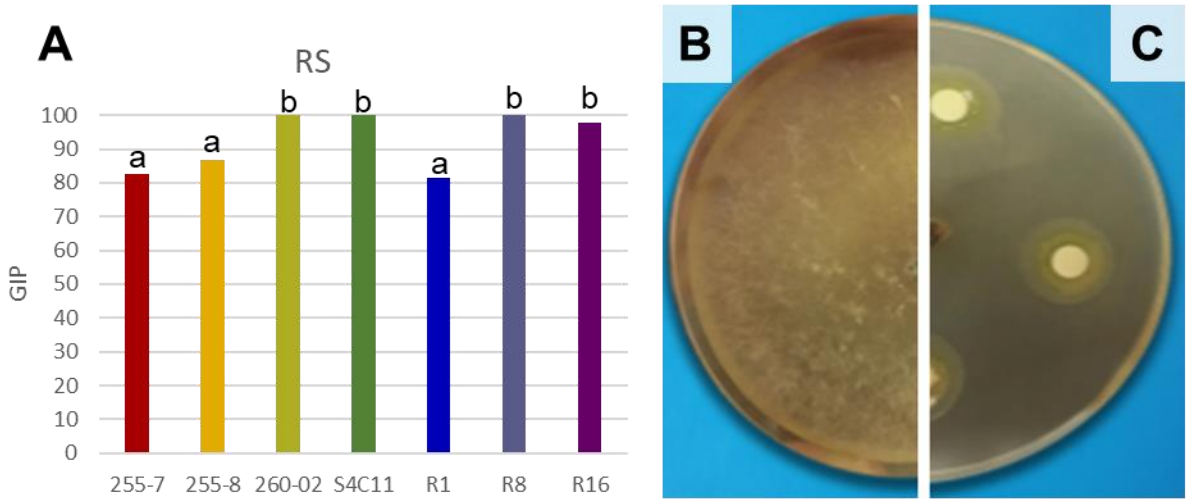


Figure 7 Results obtained from dual-culture assays regarding *Rhizoctonia solani* (RS) after 14 days of co-culture. **A)** graph reporting the GIP obtained with the different bacterial strains. Different letters indicate statistically significantly different results according to one-way ANOVA, followed by Tukey's exact test post-hoc ($p < 0.05$); **B)** picture of a non-treated control plate, showing the growth of the fungus on TGYA medium; **C)** example of a treated plate, containing strain 260-02, showing effect of dual-culture on RS: complete absence of mycelial growth;

4.1.1.6 Overall dual-culture effect

Calculating an average effect between the one displayed in the dual-culture assays against the 5 phytopathogenic fungi, it is possible to see that the strain showing the most effect against this range of fungi is strain R16, followed by strains 255-8 and R1 which show very similar values, strain 260-02, strains S4C11 and R8 which show very similar values, and lastly strain 255-7, which had the least overall effect of inhibition (**Figure 8**).

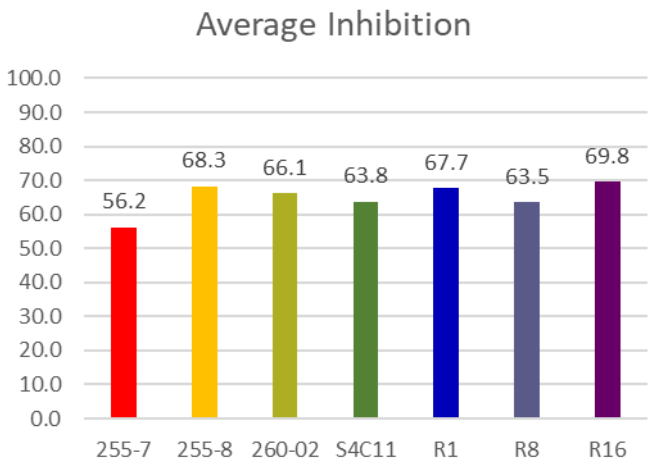


Figure 8 Graph displaying the average GIP obtained among the dual-culture assays carried out against AN, BC, FV, PV, and RS

4.1.2 Dual-plate assays

Dual-plate assays allowed to determine whether the 7 bacterial strains could inhibit the growth of phytopathogenic fungi through the production of volatile molecules only, without having growth medium continuity with the fungus. This assay was carried out on 3 fungi: BC, FV, and PV.

4.1.2.1 Inhibition of *Botrytis cinerea* mediated by VOCs

All 7 bacterial strains showed the ability to reduce the growth of BC in dual-plate assays, with an effect ranging between approximately 50% (strain 255-8 GIPV = 55) and over 90% (strain 260-02 GIPV = 92) (**Figure 9**).

It is of note that only one strain, R8, had a comparable effect in both dual-plate and dual-culture assay (GIP = 78, GIPV = 72). In the case of strain 260-02, the GIPV value is instead 28 points higher than the GIP (GIP = 64, GIPV = 92). For the 5 other strains, the GIPV value is from 12 (S4C11, R1) to 45 (255-8) points lower than the GIP registered against BC.

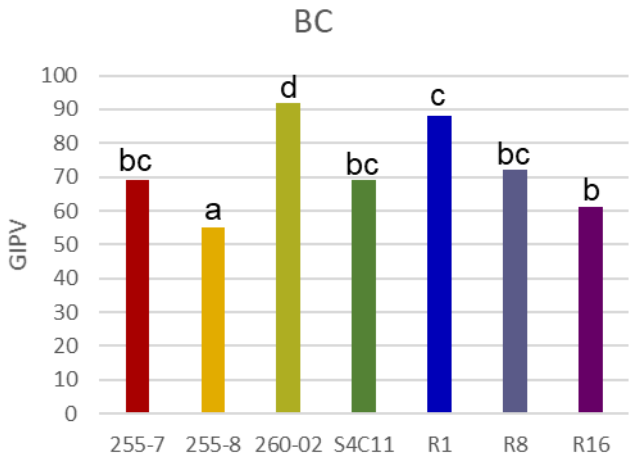


Figure 9 The graph reports the results obtained from dual-plate assays regarding *Botrytis cinerea* (BC), showing the growth inhibition percentage caused by volatile molecules (GIPV) obtained after 14 days of co-culture with the different bacterial strains. Different letters indicate statistically significantly different results according to one-way ANOVA, followed by Tukey's exact test post-hoc ($p < 0.05$).

4.1.2.2 Inhibition of *Fusarium verticillioides* mediated by VOCs

Similarly to the dual-culture assay, also the dual-plate assay evidenced no effect on the growth of FV (data not shown). Also strain 260-02, which managed to exert a slight reduction of growth in the fungal colony during dual-culture assays, had no effect on FV in the conditions of dual-plate assay.

4.1.2.3 Inhibition of *Phomopsis viticola* mediated by VOCs

Out of the 7 bacterial strains, 6 managed to reduce the growth of PV during dual-plate assays. Strain 255-8 had no effect on the growth of PV, while the other 6 strains managed to reduce the growth of the fungus with an inhibition ranging between 5% (strain R16) and 53% (strain 260-02) (**Figure 10**).

Regardless of the actual inhibition value, all bacterial strains registered a less effective inhibition against PV in dual-plate assay, compared to that obtained during dual-culture.

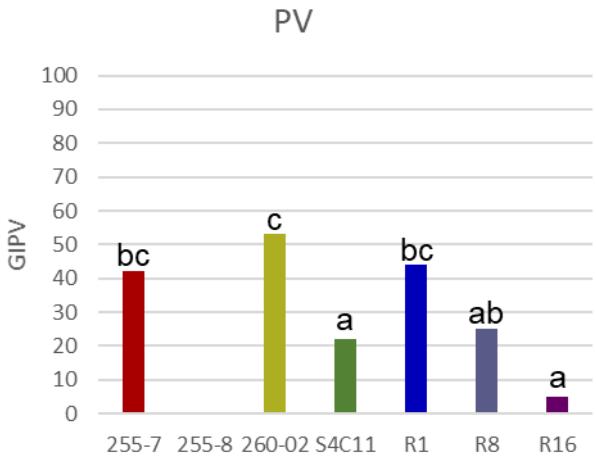


Figure 10 The graph reports the results obtained from dual-plate assays regarding *Phomopsis viticola* (PV), showing the GIPV obtained after 14 days of co-culture with the different bacterial strains. Different letters indicate statistically significantly different results according to one-way ANOVA, followed by Tukey's exact test post-hoc ($p < 0.05$).

4.1.3 Characterization of volatile compounds produced by candidate BCA strains

The characterization of volatiles produced by the 7 bacterial strains, with or without presence of BC, identified 26 molecules in total that were above detection threshold (more than 2 times the base noise).

The first analysis made on the profiles of volatiles was to determine whether there were differences in the profiles of the strains growing alone or in the presence of the pathogenic fungus BC. Statistical analyses (one-way ANOVA) identified no statistical differences between the abundance of each volatile molecule in the samples in which the strains grew alone and those in which BC was present. For brevity, these results are reported only for strain 255-7 (**Table 5**).

Since these treatments were not different, the variable of whether BC was present together with the strain or not was not considered in further analyses, reducing the analyzed treatments to the growing medium alone, BC alone, and each strain growing alone.

Out of all the 26 identified molecules, 13 could be detected in two or more of the profiles (**Table 6**), while 13 were unique to a single strain that produced them (**Table 7**).

Eleven out of 13 of these common molecules (3-methyl-butanal, acetone, 2-butanone, 2-pentanone, ethanol, 3-methyl-1-butanol, 2-methyl-1-butanol, phenyl-ethyl alcohol, dimethyl disulfide, and benzaldehyde) are present also in the profile of the sterile terrain, meaning that they are likely produced as a byproduct of the preparation and heating of the TGYA terrain and are not produced by the strains.

Of these 11 compounds, 5 seem to be exclusively present in the medium and can be in some cases degraded by the growth of the microorganisms, since there is no strain which has a higher presence of these compounds than the TGYA samples, but some have a lower value for these compounds, or they are completely absent: 3-methyl-butanal, acetone, 2-butanone, 2-methyl-1-butanol, and benzaldehyde.

The other 6 can be produced by some strains, as their abundance is significantly higher in the samples of those strains: 2-pentanone (produced by 260-02), ethanol (produced by 255-7 and R8), 3-methyl-1-butanol (produced by 255-7), 1-butanol (produced by 255-7, 255-8, R1, R8, and R16), phenyl-ethyl alcohol (produced by 255-7), and dimethyl disulfide (produced by 255-8).

The last 2 molecules present in multiple profiles are 2-heptanone (produced by 255-8 and 260-02), and 3-OH-2-butanone (produced by 255-7, 255-8, and R1). In neither of these cases there are significant differences between the production of the different strains.

It is of note that strain S4C11 and BC show no presence of molecules which are not found in the medium alone, suggesting a very low production of volatile molecules.

The 13 molecules identified only in single strains are found in strain 255-7 (cyclodecane and cyclododecane), strain 255-8 (2-heptanol), strain 260-02 (1-heptyl-2-methyl cyclopropane, methyl isobutyl ketone, 2-nonanone, 2-undecanone, 2-nonanol, 1-4-octadiene, cyclodecene), and strain R16 (isobutyl styryl ketone, 1,1-dimethyl-2-[4-(5-nitrofuran-2-yl)-1,3-thiazol-2-yl]hydrazine (DMNT), and farnesol).

The overall diversity among the volatile profiles of the strains was evaluated by a principal component analysis, in which the samples formed clusters according to their treatment, as expected (**Figure 11**). In particular, also this analysis confirms that the profiles of BC and S4C11 overlap with that of sterile TGYA, while R8 is very close to TGYA and BC but not exactly the same. Strain 255-7, 255-8, 260-02, R1 and R16 are clearly separated from the medium, indicating a consistent production of molecules, and also between themselves, accounting for the diverse production of volatile compounds of these strains.

Table 5 Table describing the presence and relative abundance of VOCs, expressed in logarithmic scale, identified in blank samples (TGY), *B. cinerea* MG53 samples (BC), strain 255-7 samples (255-7) and samples containing both strain 255-7 and BC (255-7 + BC). For each compound, name, quant ion identified through mass spectrography and retention time in our experimental conditions are reported. Values sharing the same letter (a to c) are statistically indistinguishable according to one-way ANOVA test, followed by Tukey's exact test post-hoc ($p < 0.05$).

Compound	Quant Ion	Retention Time	TGY	BC	255-7	255-7 + BC
3-methyl butanal	41	6.89	5.76	5.57	4.42	4.47
Acetone	43	5.35	5.19	5.59	0	0
2-butanone	43	6.87	5.05	5.02	0	0
2-pentanone	43	9.45	4.44	0	0	0
Ethanol	45	7.41	3.83 [a]	4.90 [a]	5.96 [b]	5.98 [b]
3-OH-2-butanone	45	22.82	0	0	5.35 [a]	5.09 [a]
Cyclodecane	55	28.80	0	0	5.03 [a]	4.96 [a]
Cyclododecane	55	32.74	0	0	5.06 [a]	5.06 [a]
3-methyl-1-butanol	55	15.50	5.80 [a]	6.26 [ab]	7.09 [b]	7.15 [b]
1-butanol	56	13.86	3.81 [a]	4.54 [ab]	6.04 [bc]	6.02 [c]
2-methyl-1-butanol	57	15.45	5.03	5.68	0	0
Phenyl-ethyl alcohol	91	32.04	5.62 [a]	5.88 [ab]	6.74 [b]	6.99 [b]
Dimethyl disulfide	94	11.20	5.20	5.78	5.18	5.25
Benzaldehyde	105	23.92	6.42 [a]	4.04 [ab]	2.55 [b]	2.86 [b]

Table 6 Table describing the presence and relative abundance of VOCs, expressed in logarithmic scale, identified in blank samples (TGY), *B. cinerea* MG53 samples (BC), strain 255-7 samples, strain 255-8, strain 260-02 samples, strain S4C11 samples, strain R1 samples, strain R8 samples, and strain R16 samples. Compounds reported in this table are common to two or more bacterial strains. For each compound, name, quant ion identified through mass spectrography and retention time in our experimental conditions are reported. Values sharing the same letter (a to c) are statistically indistinguishable according to one-way ANOVA test, followed by Tukey's exact test post-hoc ($p < 0.05$).

Compound	Quant Ion	Retention Time	TGY	BC	255-7	255-8	260-02	S4C11	R1	R8	R16
3-methyl butanal	41	6.89	5.76 ^{a}	5.57 ^{a}	4.44 ^{ab}	1.92 ^{b}	0	4.29 ^{ab}	3.20 ^{ab}	4.86 ^{ab}	5.23 ^{a}
Acetone	43	5.35	5.19 ^{a}	5.59 ^{a}	0	5.07 ^{a}	5.76 ^{a}	5.94 ^{a}	5.74 ^{a}	0	0
2-butanone	43	6.87	5.05 ^{a}	5.02 ^{a}	0	0	5.67 ^{a}	5.75 ^{a}	0	0	0
2-pentanone	43	9.45	4.44 ^{a}	0	0	0	5.90 ^{b}	0	0	0	0
2-heptanone	43	18.73	0	0	0	6.05 ^{a}	5.30 ^{a}	0	0	0	0
Ethanol	45	7.41	3.82 ^{a}	4.89 ^{a}	5.97 ^{b}	0	0	0	0	5.63 ^{b}	0
3-OH-2-butanone	45	22.82	0	0	5.23 ^{a}	6.20 ^{a}	0	0	6.15 ^{a}	0	0
3-methyl-1-butanol	55	15.50	5.80 ^{a}	6.26 ^{ab}	7.12 ^{b}	0	5.80 ^{a}	4.93 ^{a}	4.82 ^{a}	5.67 ^{a}	5.74 ^{a}
1-butanol	56	13.86	3.81 ^{a}	4.54 ^{ab}	6.04 ^{bc}	5.70 ^{bc}	0	4.81 ^{ab}	5.47 ^{bc}	5.21 ^{bc}	5.67 ^{bc}
2-methyl-1-butanol	57	15.45	5.03 ^{a}	5.68 ^{a}	0	0	5.23 ^{a}	4.27 ^{a}	0	0	5.70 ^{a}
Phenyl-ethyl alcohol	91	32.04	5.62 ^{a}	5.88 ^{ab}	6.88 ^{b}	5.35 ^{a}	5.42 ^{a}	4.91 ^{a}	4.68 ^{a}	5.22 ^{a}	5.38 ^{a}
Dimethyl disulfide	94	11.20	5.20 ^{a}	5.78 ^{ab}	5.21 ^{a}	6.73 ^{b}	5.90 ^{ab}	5.22 ^{a}	4.73 ^{a}	5.31 ^{a}	4.88 ^{a}
Benzaldehyde	105	23.92	6.42 ^{a}	4.04 ^{ab}	2.73 ^{b}	2.16 ^{b}	3.76 ^{b}	2.30 ^{b}	2.36 ^{b}	3.37 ^{b}	4.99 ^{ab}

Table 7 Table describing the presence and relative abundance of VOCs, expressed in logarithmic scale, identified in strain 255-7 samples, strain 255-8, strain 260-02 samples, strain S4C11 samples, strain R1 samples, strain R8 samples, and strain R16 samples. Compounds reported in this table are unique to only one of the seven bacterial strains. For each compound, name, quant ion identified through mass spectrography and retention time in our experimental conditions are reported.

Compound	Quant Ion	Retention Time	255-7	255-8	260-02	S4C11	R1	R8	R16
1-heptyl-2-methyl cyclopropane	41	12.78			6.75				
Methyl isobutyl ketone	43	10.82			5.41				
2-nonanone	43	25.89			5.42				
2-undecanone	43	31.57			4.56				
Isobutyl styryl ketone	43	40.15							6.25
2-heptanol	45	18.54		4.87					
2-nonanol	45	29.54			5.08				
Cyclodecane	55	28.80	4.99						
Cyclododecane	55	32.74	5.06						
1-4-octadiene	67	18.19			5.2				
Cyclodecene	67	25.11			4.20				
DMNT	69	17.98							6.18
Farnesol	69	29.72							7.03

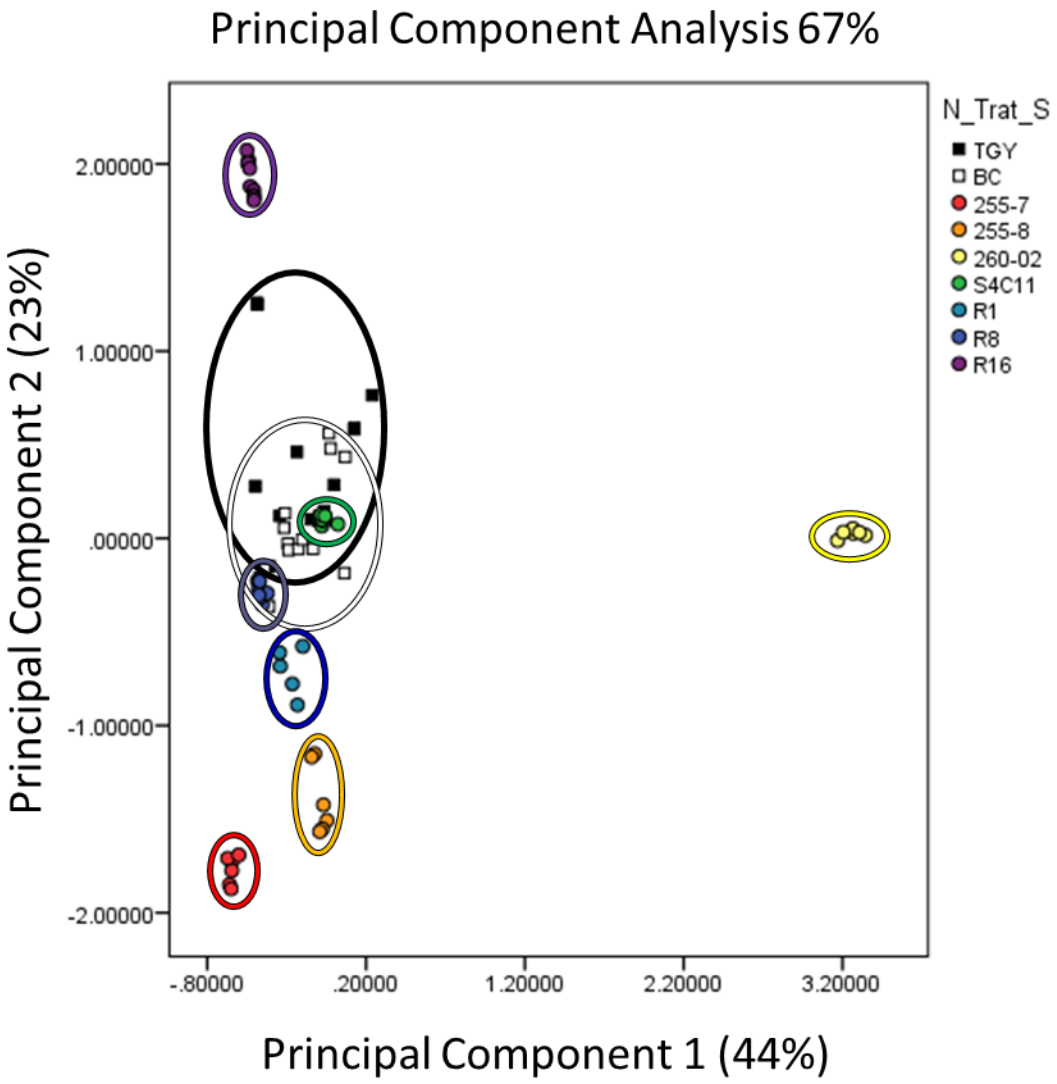


Figure 11 Scatter plot describing the principal component analysis (explaining 67% of variation) carried out on the base 10 logarithmic values of the relative quantities of volatile compounds identified in the different samples. Each sample is reported as a dot, while circles describe the grouping of samples based on the type of sample (TGYA medium only, *B. cinerea* MG53, or one of the 7 bacterial strains).

4.1.4 Inhibition of *Botrytis cinerea* growth by cultural filtrate

This assay determined whether the 7 bacterial strains produced diffusible molecules characterized by an antifungal effect against BC.

All the strains demonstrated an effect, inhibiting the mycelial growth of BC: apart from strain R16, that determined only 56% of GIPf, all the strains showed values of GIPf close to 100 (**Figure 12**).

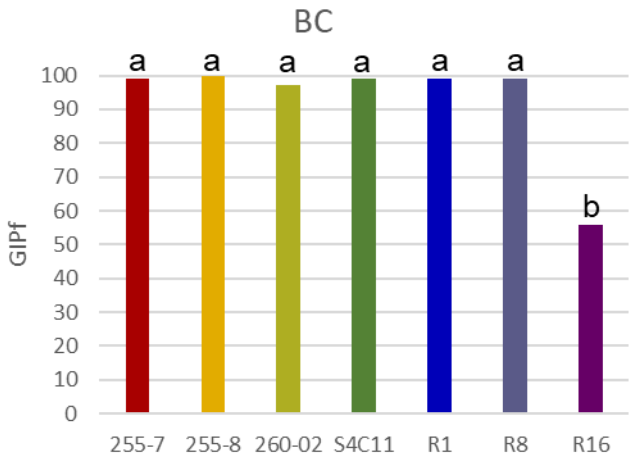


Figure 12 The graph reports the results obtained from assays carried against *Botrytis cinerea* (BC), out with TGYA plates in which cultural filtrate of the various bacterial strains was mixed in the medium. The graph shows the growth inhibition percentage caused by cultural filtrate (GIPf) obtained after 14 days of incubation. Different letters indicate statistically significantly different results according to one-way ANOVA, followed by Tukey's exact test post-hoc ($p < 0.05$).

4.1.5 Inhibition of *B. cinerea* spore germination by co-culture

This assay determined whether the bacterial strains could inhibit the germination of BC conidia, preventing development at a previous stage compared to mycelial growth.

The non-treated BC samples showed only a low level of germination after 6 hours of incubation, reaching a peak of germination rate (GR) of almost 90% after 18 hours, and remaining close to 80% after 72 hours (**Figure 13A**).

Bacterial strains 255-7, R1, and R8 showed a weak effect in inhibiting the germination of the conidia: strain R8 reduced the germination throughout the whole duration of the assay, bringing GR down to 60% at 18 hours and keeping it to 67% after 72 hours; strain R1 had a similar effect, showing a more effective inhibitory effect during the first 48 hours, but then allowing the conidia to germinate to a rate similar to the non-treated controls after 72 hours; likewise, strain 255-7 completely inhibited the germination during the first 24 hours, but then started to lose efficacy, allowing conidia to germinate, albeit at a lower rate compared to the non-treated control.

The other four strains (255-8, 260-02, S4C11, R16) had an almost complete inhibitory effect on the germination of conidia and, among them, it is of note that in the samples treated with strain S4C11 spores appeared to become degraded by the bacteria, as if being actively lysed by the bacterial cells (**Figure 13C**).

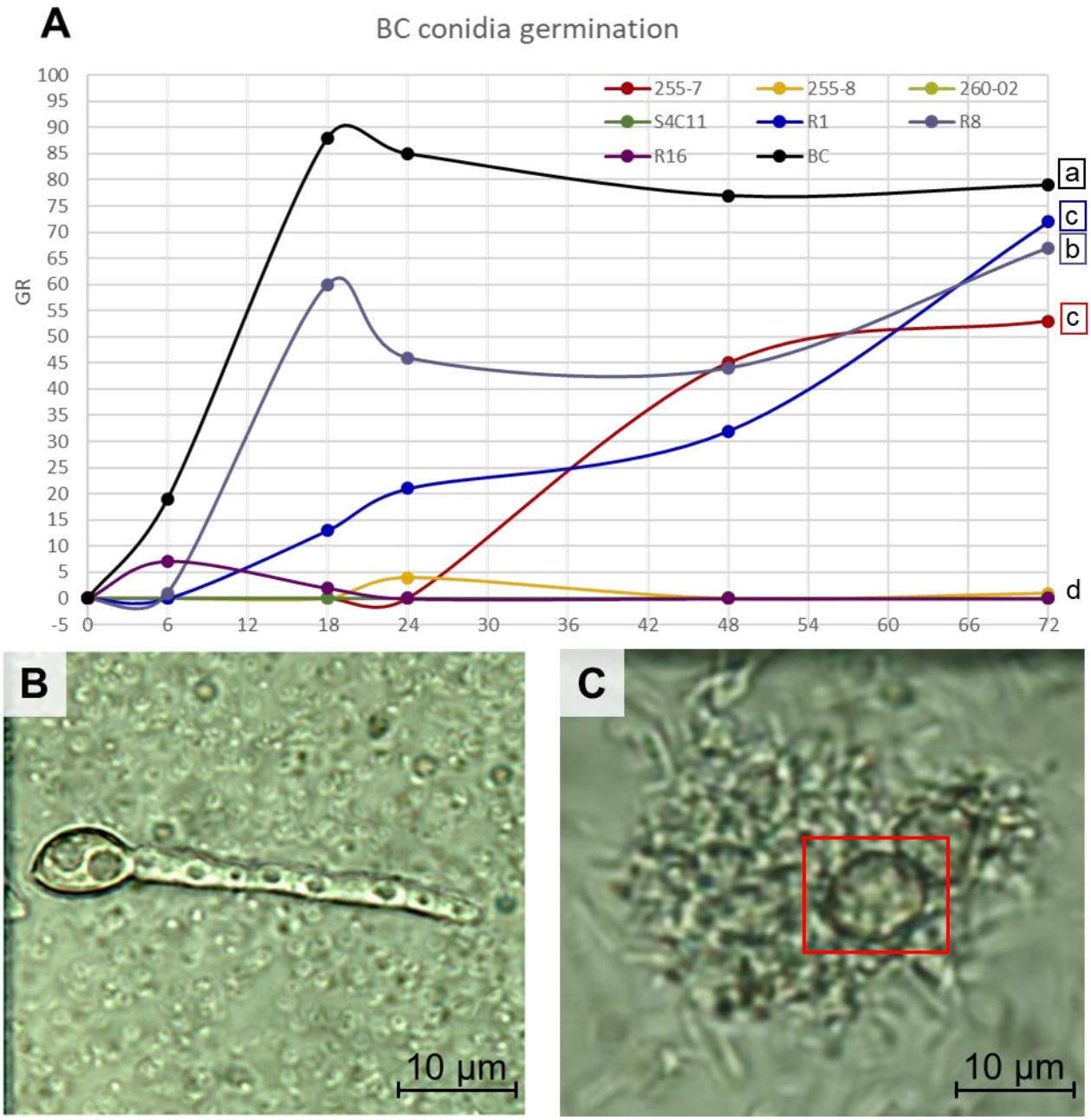


Figure 13 Results obtained from co-culture assay on germination of BC conidia. **A)** the graph shows on the Y axis germination rates (GR), expressed as percentage, of the non-treated control and of the samples treated with the 7 bacterial strains through time (hours, on the X axis). Different letters (a to d) indicate statistically significantly different results throughout the 72 hours according to one-way ANOVA, optimized for repeated measures, followed by Tukey's exact test post-hoc ($p < 0.05$); **B)** germination of a non-treated conidium; **C)** damaged conidia detected in samples treated with strain S4C11, highlighted by the red box.

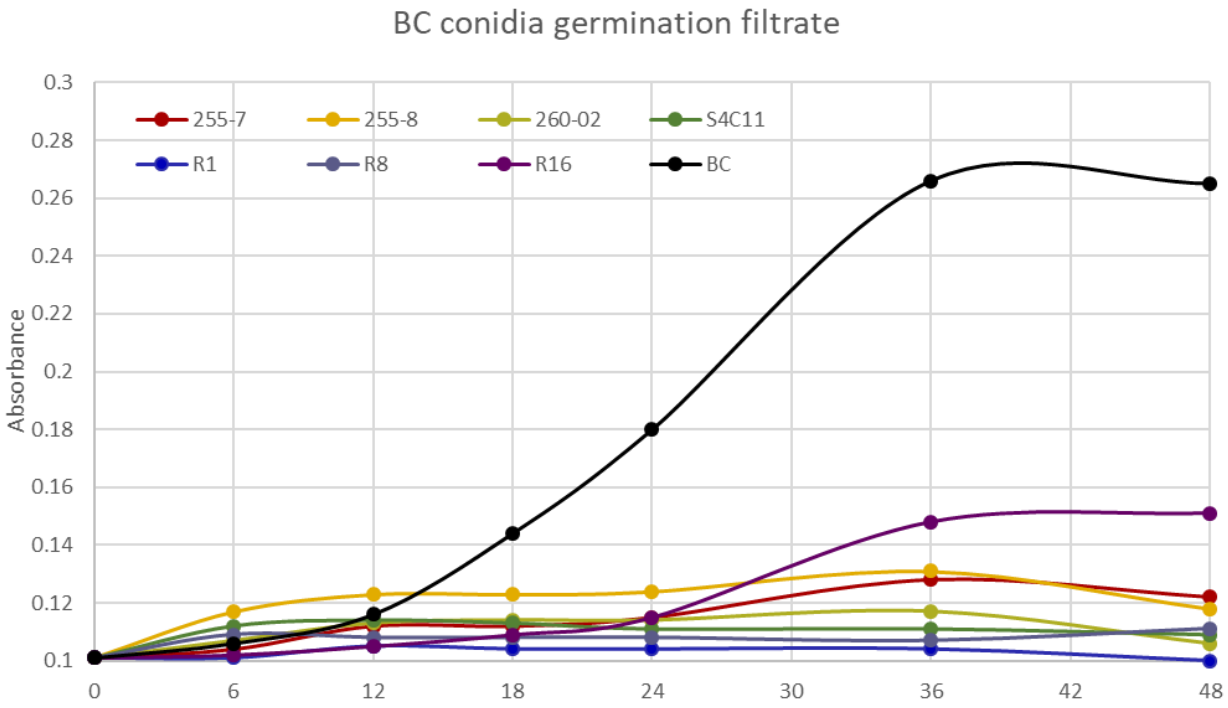
4.1.6 Inhibition of BC spore germination by cultural filtrate

This assay determined whether the cultural filtrate of the bacterial strains could inhibit the germination of BC conidia.

Similarly to the what observed in the previous assays, also in this case the germination of conidia is first observed in the non-treated samples after 12 hours of incubation and has a significant increase starting from 18 hours.

All the cultural filtrates had an inhibiting effect on the germination of conidia, as shown by the little, or absent, increase in absorbance in the treated samples (**Figure 14**), with the only strain R16 seeming to have a delaying effect, rather than inhibiting the germination of conidia, since the absorbance measured after 48 hours is perfectly in line to that exhibited by the non-treated controls after 18 hours.

More specific assays were carried out using cultural filtrates treated with proteinase K and heat treatment in autoclave were used to determine which kind of molecules could be involved in the inhibition. These two assays were not carried out with strain R16, which filtrate had too little an effect even without undergoing further treatments.



	6	12	18	24	36	48	Stat
TNT	0.106	0.115	0.143	0.145	0.266	0.265	a
255-7	0.103	0.112	0.112	0.106	0.128	0.124	bc
255-8	0.114	0.118	0.121	0.115	0.131	0.118	b
260-02	0.106	0.113	0.113	0.106	0.117	0.106	c
S4C11	0.112	0.113	0.112	0.110	0.111	0.109	c
R1	0.101	0.105	0.105	0.099	0.104	0.099	d
R8	0.108	0.108	0.108	0.109	0.107	0.110	c
R16	0.101	0.105	0.108	0.11	0.147	0.15	b

Figure 14 Results obtained from the germination assay carried out on BC conidia incubated in TGY medium, or TGY added with cultural filtrates of the different bacterial strains. The graph shows on the Y axis the absorbance (492 nm wavelength) measured throughout 48 hours of incubation (X axis), while the average values (7 replicates) are reported in the table. The Stat column reports the results of the one-way ANOVA test, optimized for repeated measures, followed by Tukey's exact post-hoc test ($p < 0.05$). Different letters indicate significantly different results throughout the whole 48 hours experiment.

4.1.6.1 Conidia inhibition by strain 255-7 cultural filtrate

The inhibitory effect of the filtrate from strain 255-7 shows to lose some efficacy when treated with protease K, in which case the effect seems to be a mere delaying of germination rather than inhibition, while the filtrate treated with heat shows no difference when compared to the non-treated filtrate (**Figure 15A**), suggesting that all the inhibitory components are thermostable and that some of them may be peptides.

4.1.6.2 Conidia inhibition by strain 255-8 cultural filtrate

The cultural filtrate of strain 255-8 loses much of its inhibitory capacity when treated with either proteinase K or heat, as in both cases the absorbance measured at 48 hours of incubation is comparable to that registered at 24 hours in the non-treated controls, and both values are significantly higher than those registered for the non-treated filtrate (**Figure 15B**), indicating that both peptides and thermosensitive components are involved in the inhibition.

4.1.6.3 Conidia inhibition by strain 260-02 cultural filtrate

The cultural filtrate of strain 260-02 loses much of its inhibitory capacity when treated with either proteinase K or heat, as in both cases the absorbance measured at 48 hours of incubation is comparable to that registered at 24 hours in the non-treated controls, and both values are significantly higher than those registered for the non-treated filtrate (**Figure 15C**) , indicating that both peptides and thermosensitive components are involved in the inhibition.

4.1.6.4 Conidia inhibition by strain S4C11 cultural filtrate

The cultural filtrate of strain S4C11 behaves differently than all others: while there is a significant difference between the effects obtained with either the proteinase K-treated filtrate or the heat-treated filtrate and the non-treated one, the inhibitory effect remains, preventing the germination of conidia even after 48 hours of incubation (**Figure 15D**), indicating that while some components involved in the inhibition are peptides and/or thermosensitive, the main mechanism relies on other sorts of molecules.

4.1.6.5 Conidia inhibition by strain R1 cultural filtrate

The cultural filtrate of strain R1 becomes less effective when treated with heat, and even less effective when treated with proteinase K (**Figure 15E**). In this last case, the absorbance measured at 48 hours of incubation is even higher than that registered at 48 hours in the non-treated control, signaling not only a complete germination, but also a production of mycelium. This result indicates that the peptides in the filtrate are presumably more important than the thermosensitive components in achieving the inhibition.

4.1.6.6 Conidia inhibition by strain R8 cultural filtrate

The inhibitory effect of the filtrate from strain R8 shows to lose efficacy when treated with protease K, in which case the effect seems to be a mere delaying of germination rather than inhibition, while the filtrate treated with heat shows little difference from the non-treated filtrate (**Figure 15F**), suggesting that most of the inhibitory components are thermostable and that some of them may be peptides.

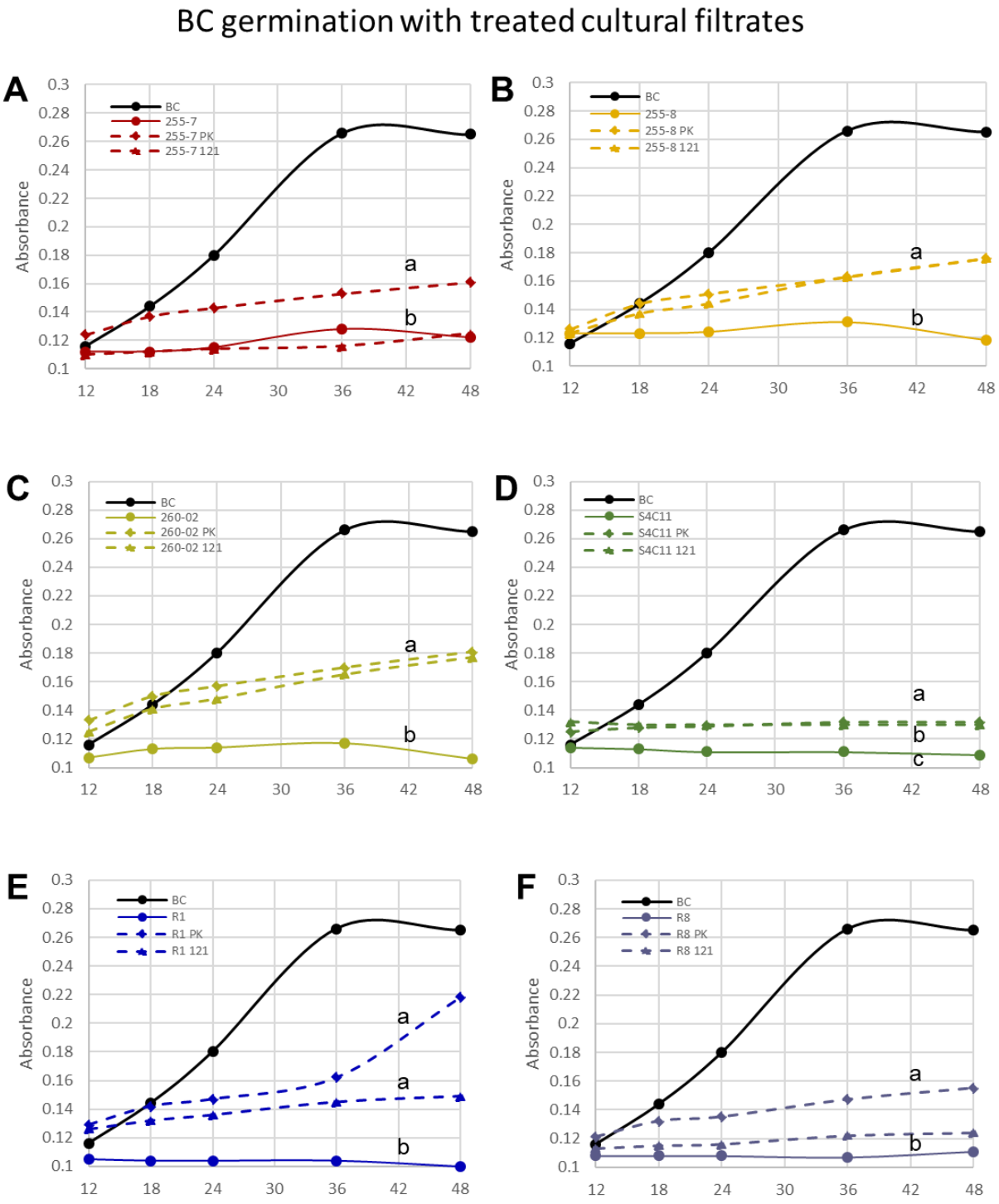


Figure 15 Results obtained from the germination assay carried out on BC conidia incubated in TGY medium, or TGY added with cultural filtrates of the different bacterial strains, either non-treated, treated with proteinase K, or by autoclaving. The graphs show on the Y axis the absorbance (492 nm wavelength) measured throughout 48 hours of incubation (X axis) for **A)** strain 255-7; **B)** strain 255-8; **C)** strain 260-02; **D)** strain S4C11; **E)** strain R1; **F)** strain R8. Different letters indicate statistically significantly different results throughout the 48 hours according to one-way ANOVA, optimized for repeated measures, followed by Tukey's exact test post-hoc ($p < 0.05$).

4.2 Results of *in vivo* assays

In this chapter are reported the results obtained in the biocontrol assays carried out in *in vivo* conditions against *B. cinerea* strain MG53 (BC).

4.2.1 *In vivo* biocontrol of *B. cinerea* on tomato berry

This assay determined whether the 7 bacterial strains could inhibit the development of gray mold disease on tomato berries in postharvest conditions, when the disease manifests itself in real applicative conditions.

Strain MG53 of *B. cinerea* demonstrated itself to be very aggressive on the tomato berries, developing severe symptoms on the control berries that were inoculated only with the pathogen (**Figure 16A**), determining class 6 symptoms on 129 out of 210 berries (61%), and the presence of residual *B. cinerea* inoculum from before the sterilization can be excluded by the lack of symptoms in the non-treated control berries.

While all 7 strains managed to reduce the colonization index (C%I) of the pathogen on the berries, the effect of strain 255-8 and R16 is not significant, as the C%I of berries treated with these strains is not statistically different from that of berries inoculated only with BC. In these berries, though, only 8 (27%) and 6 (20%) berries, respectively, out of 30 showed class 6 symptoms.

The other 5 strains instead showed an appreciable decrease in the colonization index, ranging between almost 75% (strain S4C11 C%I = 75.9) and almost 50% (strain 255-7 C%I = 51.8), compared to the over 90% registered on berries inoculated only with the pathogen (BC C%I = 92.1) (**Figure 17**). For this last, most effective strain, only 1 berry out of 30 (3%) showed class 6 symptoms and 4 berries (13%) showed the mildest, class1 symptoms. The most abundant symptom class was 3 with 9 out of 30 berries (30%) displaying these symptoms of medium intensity.

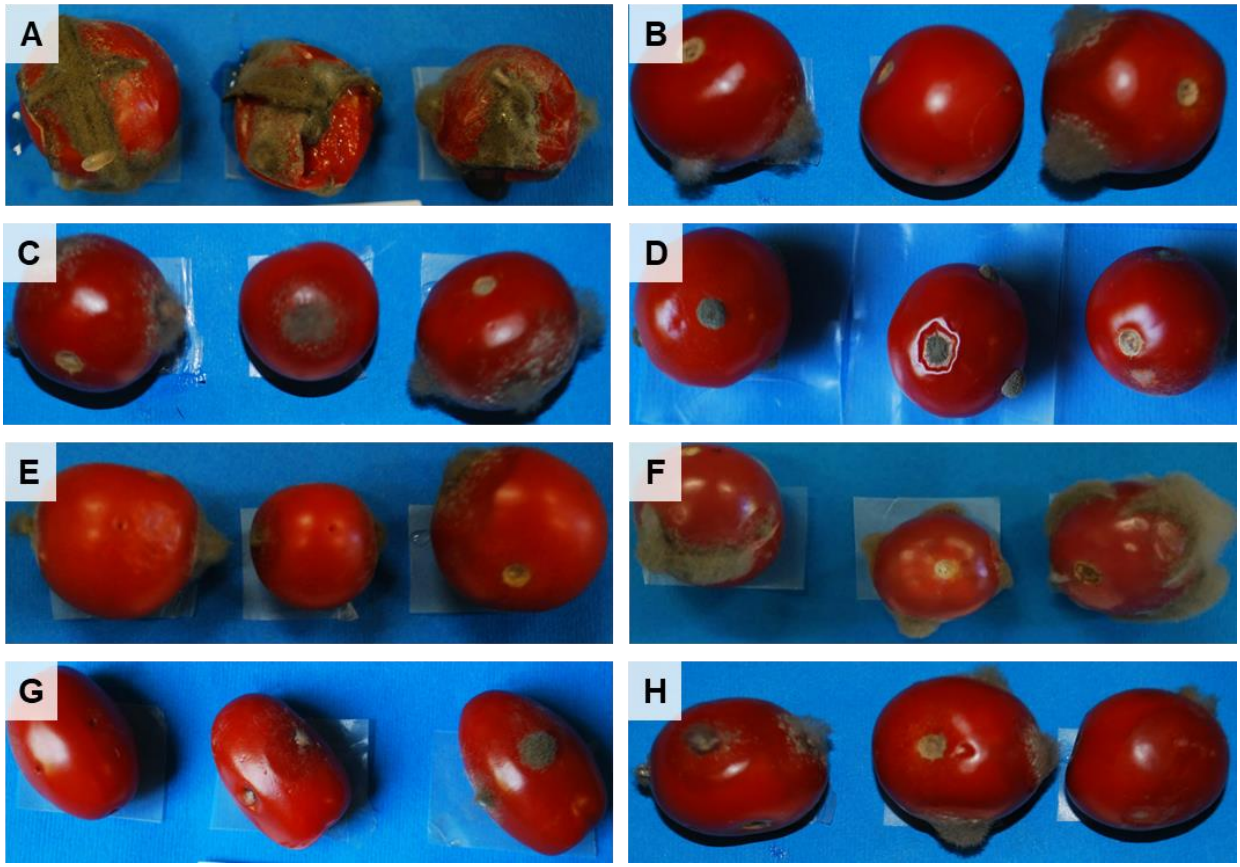


Figure 16 Photographs showing the results obtained for *in vivo* biocontrol on tomato berries against BC. The different photos show either the berries inoculated either with **A)** BC alone, or inoculated with BC after being treated with **B)** strain 255-7; **C)** strain 255-8; **D)** strain 260-02; **E)** strain S4C11; **F)** strain R1; **G)** strain R8; **H)** strain R16.

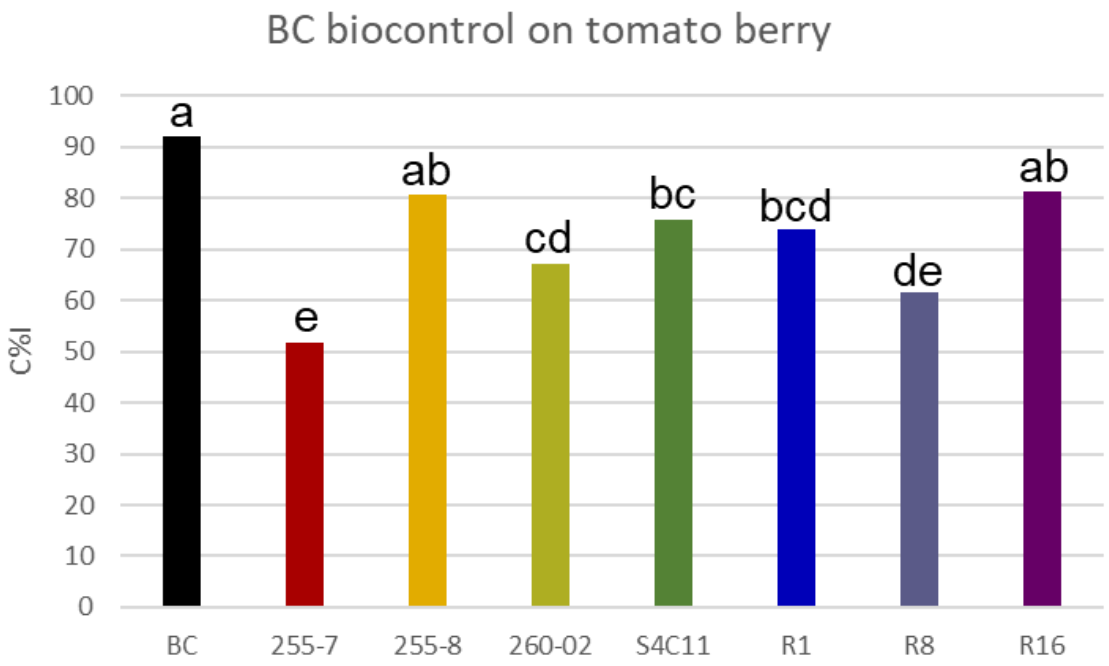


Figure 17 Graph showing the average percentage of colonization index (C%I) observed on tomato berries treated either with BC alone, or with the pathogen and one of the 7 bacterial strains (30 berries for treatment). Different letters (from a to e) indicate significant statistical difference between the results according to One-way ANOVA, followed by Tukey's exact post-hoc test ($p < 0.05$).

4.2.2 *In vivo* biocontrol of *B. cinerea* on grapevine leaves

This assay determined whether the 7 bacterial strains could inhibit the development of gray mold on the leaf tissue of grapevine since, in field conditions, the infection often starts on the leaves before moving to the berries.

Once again, BC was very effective in colonizing the leaves and determining quickly some intense symptoms on the leaves, causing them to develop brown spots on their surface (**Figure 18A**).

The measurements of infection carried out at 3, 5, and 7 days from the fungal inoculum followed the same trend for the different bacteria and, therefore, it can be said that the inhibitory effect, when present, remained consistent during the 7 days period (**Figure 18**).

Only 2 strains, 255-7 and 260-02, managed to sensibly reduce the C%I, while strains 255-8, S4C11, and R1 did reduce the colonization rate, but not enough to be significantly different from the control inoculated only with the pathogen. Instead, strains R8 and R16 had no effect on BC growth in this assay.

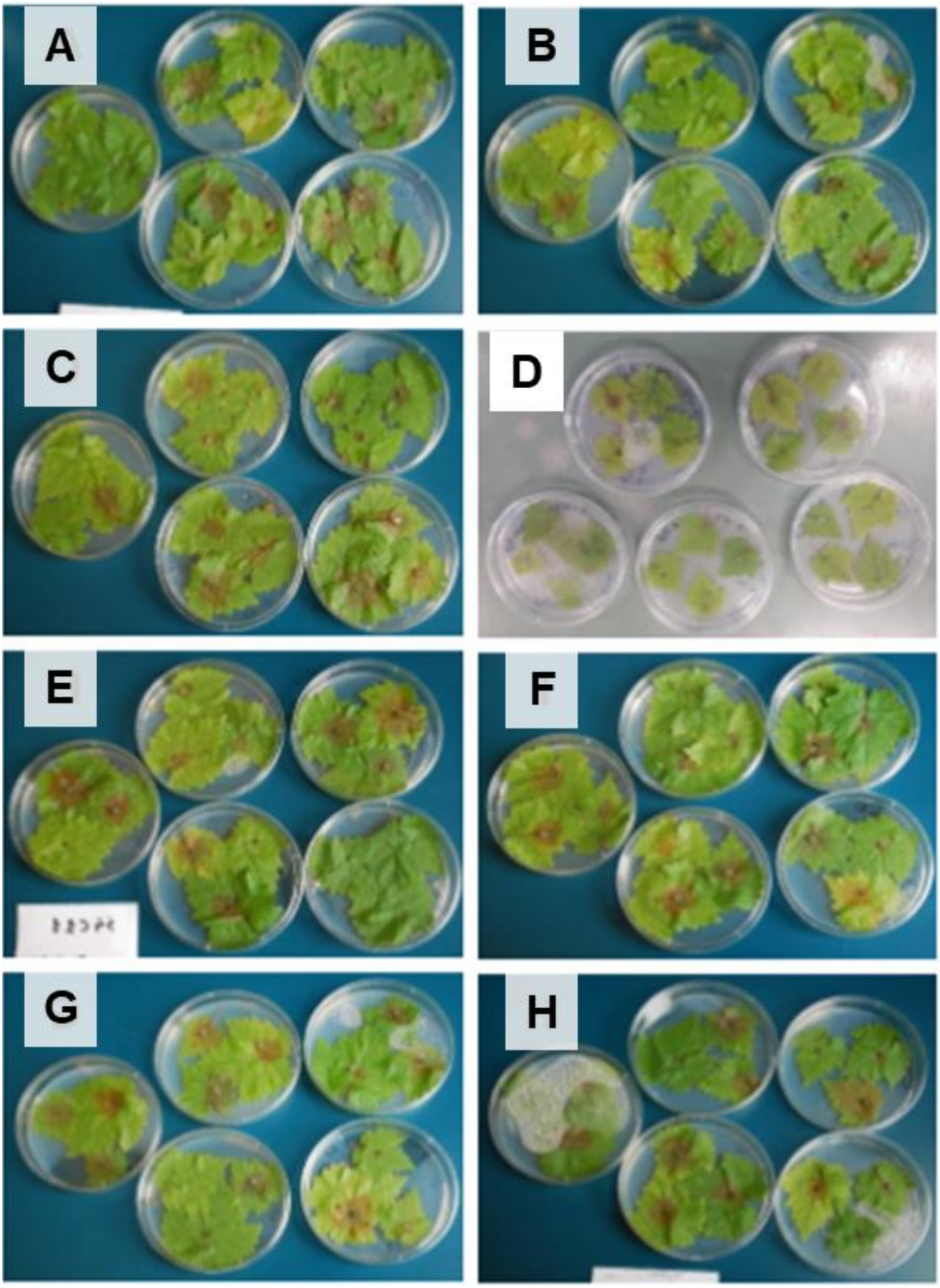


Figure 18 Photographs of the plates containing the grapevine leaves, either **A)** inoculated with the pathogen alone; or inoculated with the strains **B)** 255-7; **C)** 255-8; **D)** 260-02; **E)** S4C11; **F)** R1; **F)** R8, **H)** R16; at three days from the fungal inoculum.

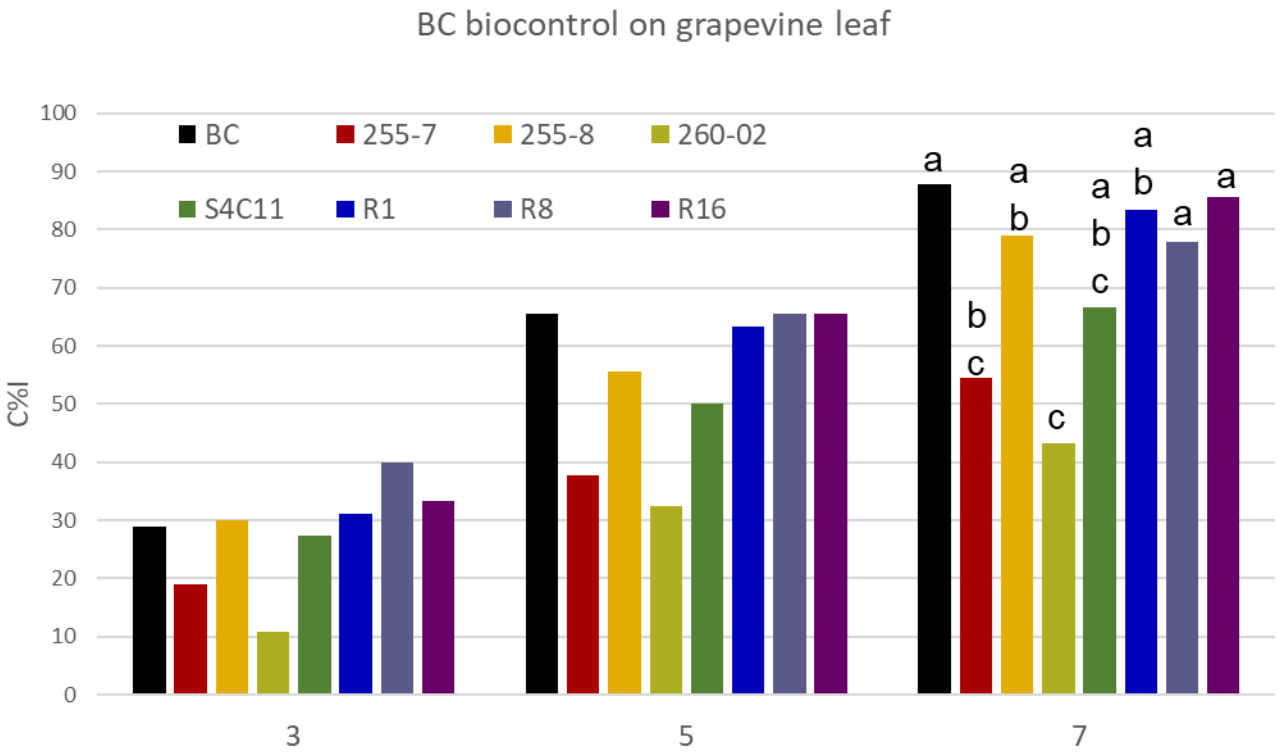


Figure 19 Graph showing on the Y axis the C%I observed on grapevine leaves treated either with BC alone, or with the pathogen and one of the 7 bacterial strains, after 3, 5, or 7 days (X axis). Different letters (from a to c) indicate significant statistical difference between the results according to One-way ANOVA, followed by Tukey's exact post-hoc test ($p < 0.05$).

4.3 Results of *in planta* assays

In this chapter are presented all the results obtained from *in planta* assays, both regarding the promotion of plant growth and the biocontrol assays.

4.3.1 Plant growth promotion assays

4.3.1.1 Pathogenicity and colonization tests

These tests were carried out to detect whether the 7 bacterial strains and the selected pathogens could cause symptoms on the leaves of some model organisms (*N. benthamiana* and *N. tabacum*), if the 7 strains could cause damage by interacting with the roots of pepper plants (Quadrato d'Asti variety), and if the 3 labeled strains (255-7:*rfp*, 260-02:*gfp*, R8::*gfp*) could effectively colonize different plant hosts.

Pathogenicity tests gave negative results for all 7 bacterial strains, with none of the inoculation methods used causing symptoms on the host plants, while inoculum of *Pseudomonas syringae* DC3000, both wild-type and transformed with *gfp*, caused the development of necrotic lesions (**Figure 20 A** and **B**). Likewise, CymRSV caused the development of necrotic lesions on the inoculated leaves (**Figure 20 C**).

The colonization assays were positive for all examined plant species: from the leaf tissues of the inoculated plants the bacterial strains were recovered on selective medium with kanamycin in all three the assayed dilutions, and the *gfp* or *rfp* gene, as appropriate, were detected by PCR amplification, while both tests were negative on non-treated control plants.

The more in-depth microscopy assay carried out for strain 260-02 clarified the pattern of epiphytic root colonization of this strain, showing that it favors primary roots and zones where new roots are emerging, while it shows little or no colonization of secondary roots or root tips, with these patterns remaining consistent in the investigated 6 days post inoculation (**Figure 21**). It is a different pattern from that demonstrated by a pathogenic strain belonging to the same species, as DC3000:gfp shows preference for secondary roots and damaged root tissue, often colonizing in an excessive way these weakened structures, but is not found in association to primary roots (**Figure 21**)

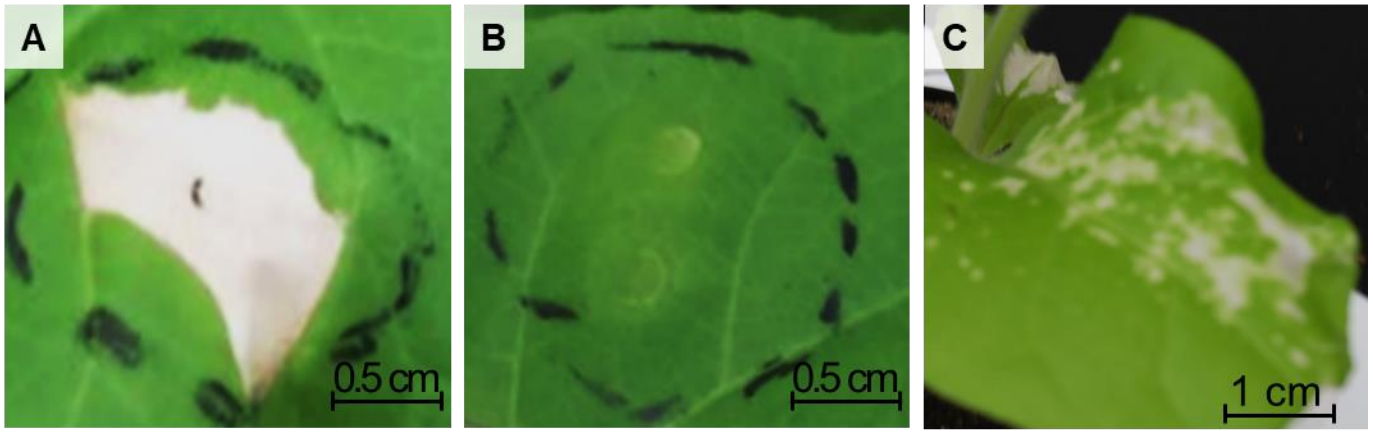


Figure 20 Photograph of *N. benthamiana* leaves 5 days after inoculation by infiltration of either cell suspension of **A)** *P. syringae* strain DC3000 or **B)** *P. syringae* strain 260-02. Development of white necrosis is visible on **A**, while only the imprint of the infiltration is visible in **B**. C) Leaf of *N. benthamiana* 7 days after mechanical inoculation of CymRSV, showing necrotic damage.

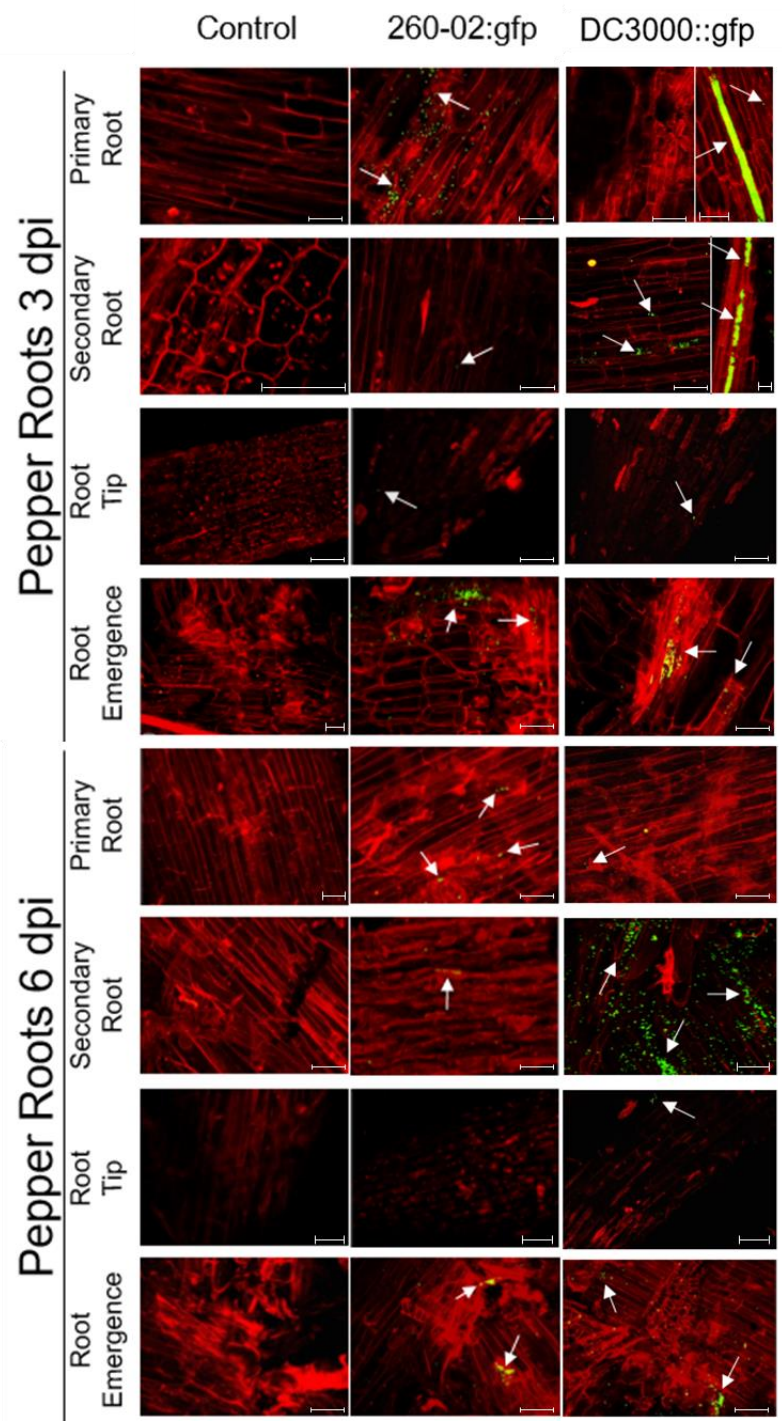


Figure 21 Panel of photographs taken by laser confocal microscopy: red indicates the structure of plant tissues, while green indicates cells of strain 260-02:*gfp* or DC3000::*gfp*, as indicated at the top of each column of pictures. White arrows highlight zones where green cells can be seen. Control pictures are shown to prove that no endogenous green fluorescence could be detected, and that the fluorescent cells come from the root dip treatment. Images are divided in two groups by the time after which they were taken (3 or 6 days) and compared between control and treatments, as well as between four different root structures (primary root, secondary root, root tip, root emergence) being compared on the vertical direction. The scale bar in each panel has the length of 15 µm.

4.3.1.2 Plant-growth promotion ability of strain 255-7:*rfp*

Strain 255-7:*rfp* (*Pantoea agglomerans*) was assayed on several plant species: bean (*Phaseolus vulgaris* var. Nano bobis), zucchini (*Cucurbita pepo* var. Genovese), cucumber (*Cucumis sativus* var. Tasty Green), tomato (*Solanum lycopersicum* var. San Marzano 2) eggplant (*Solanum melongena* var. Violetta lunga), and pepper (*Capsicum annuum* var. Zebo F1, trial C).

The effect of the inoculation on the different hosts can be summarized in three main categories:

- No effect: the treated tomato plants show a slight increase in height, which is not significantly different by the non-treated control (**Figure 22D**);
- Early effect: the treated plants of bean and zucchini showed a statistically significant difference from the non-treated control, but this difference is given by an increased growth in the first weeks, since the final height of these plants is the same (**Figure 22A** and **B**);
- Late effect: the treated plants of cucumber, eggplant, and pepper had the same growth pattern of the non-treated plants at first, but then started growing at an increased rate, determining a statistically significant difference compared to the non-treated control (**Figure 22C**, **E**, and **F**)

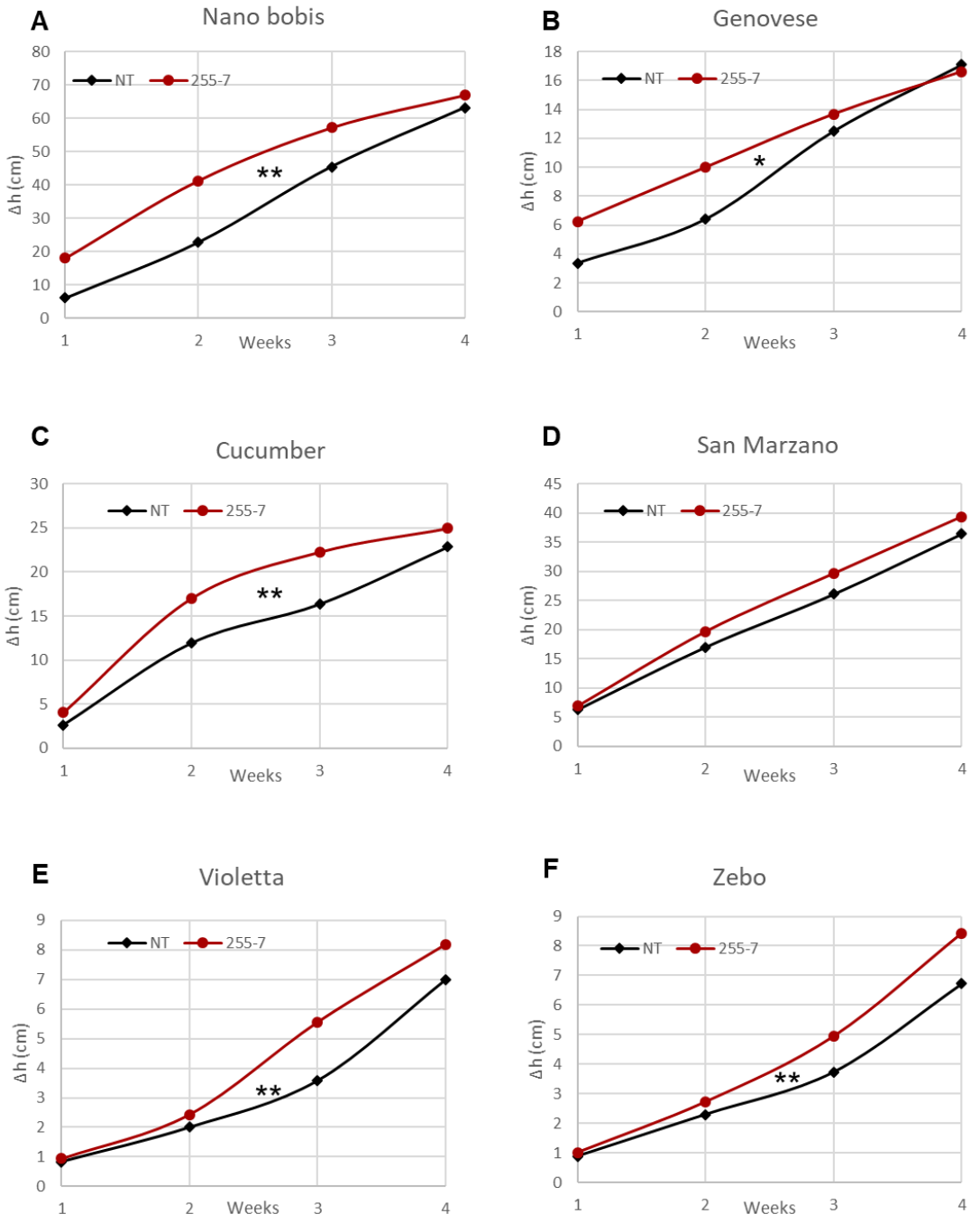


Figure 22 Results of plant-growth promotion (PGP) assays carried out with strain 255-7 on different plant species. In black is the non-treated control, while in red is the treated thesis. On the Y axis the difference in height compared to initial measurement of the plant (Δh) is reported, while on the X axis the weeks since the first measurement are reported. Stars indicate significant difference between the treated and non-treated plants throughout the observed period according to One-way ANOVA, optimized for repeated measures (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$)

4.3.1.3 Plant-growth promotion ability of strain 255-8

Strain 255-8 (*Burkholderia* sp.) was assayed on pepper (Zebo, trial B), and managed to promote the growth of the plants, compared to the non-treated control, although the difference in height is noticeable only in the last week of observation (**Figure 23A**).

Examination of the whole plant highlighted that, while the aerial part was significantly taller, the most relevant difference from the non-treated control was in the root system, which was noticeably more developed (**Figure 23B** and **C**), also, a more precocious flowering could be detected in the treated plants.

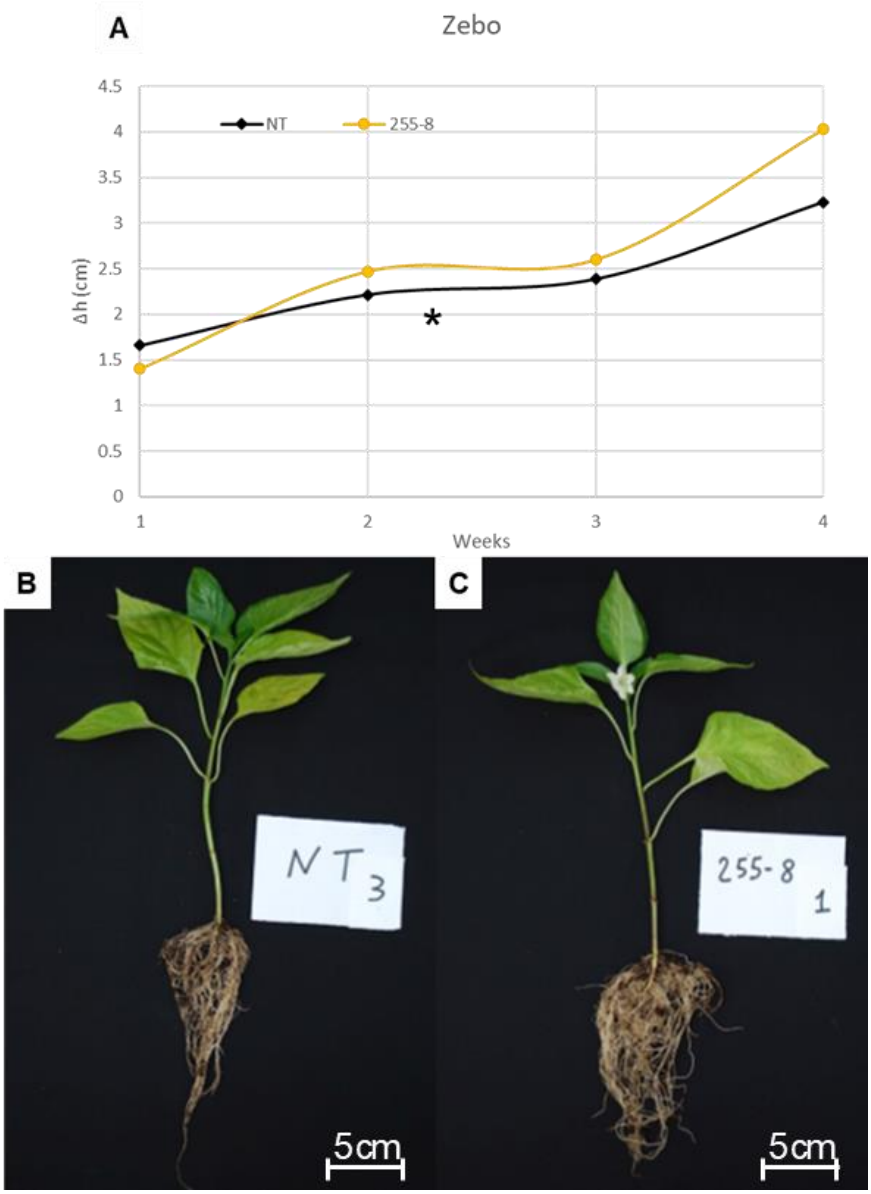


Figure 23 Results of PGP assays carried out with strain 255-8 on Zebo plants. **A)** graph reporting growth during the experiment. In black is the non-treated control, while in orange is the treated thesis. On the Y axis the Δh is reported, while on the X axis the weeks since the first measurement are reported. Stars indicate significant difference between the treated and non-treated plants throughout the observed period according to One-way ANOVA, optimized for repeated measures (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$); **B)** non-treated Zebo plant from trial B; **C)** Zebo plant treated with 255-8. The difference in root development and the presence of flower can be noticed.

4.3.1.4 Plant-growth promotion ability of strain 260-02:*gfp*

Strain 260-02:*gfp* (*Pseudomonas syringae*) was assayed on several plant species: tomato (Rio Grande and Sibari), eggplant (Beatrice), and pepper (Zebo, trial A).

The effect of the inoculation on the different hosts can be summarized in three main categories:

- No effect: the treated Rio Grande and eggplant plants showed no increase in growth and, in the case of eggplant, even a slight reduction in height, and are never significantly different by the non-treated control (**Figure 24B** and **C**);
- Early effect: the treated pepper plants showed an increase in height already from one week after inoculation, and remained consistently taller than the non-treated control throughout the experiment (**Figure 24D**);
- Late effect: the treated Sibari plants showed an increase in height over the non-treated control only in the last two weeks and, despite the little difference in actual height, this makes them statistically different from the control plants (**Figure 24A**)

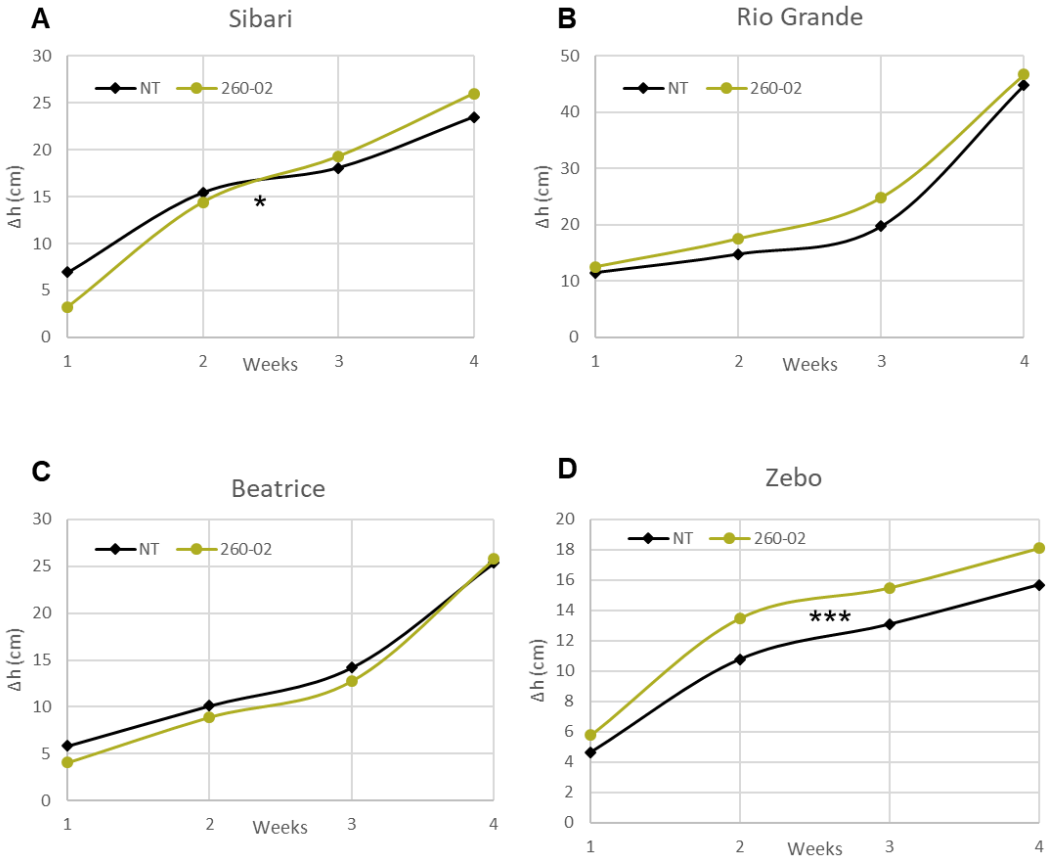


Figure 24 Results of PGP assays carried out with strain 260-02 on different plant species. In black is the non-treated control, while in yellow is the treated thesis. On the Y axis the Δh is reported, while on the X axis the weeks since the first measurement are reported. Stars indicate significant difference between the treated and non-treated plants throughout the observed period according to One-way ANOVA, optimized for repeated measures (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$)

4.3.1.5 Plant-growth promotion ability of strain S4C11

Strain S4C11 (*Lysinibacillus fusiformis*) was assayed on pepper (Zebo, trial C), and managed to noticeably promote the growth of the plants, compared to the non-treated control, over the whole duration of the experiment (**Figure 25**).

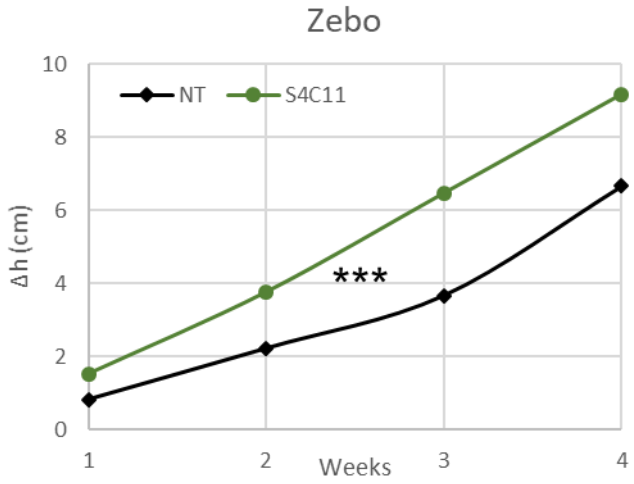


Figure 25 Results of PGP assays carried out with strain S4C11 on Zebo plants. **A)** graph reporting growth during the experiment. In black is the non-treated control, while in green is the treated thesis. On the Y axis the Δh is reported, while on the X axis the weeks since the first measurement are reported. Stars indicate significant difference between the treated and non-treated plants throughout the observed period according to One-way ANOVA, optimized for repeated measures (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$)

4.3.1.6 Plant-growth promotion ability of strain R1

Strain R1 (*Bacillus pumilus*) was assayed on pepper (Zebo, trial C), and managed to promote the growth of the plants, compared to the non-treated control, giving a slight, but consistent, growth promotion effect throughout the experiment (**Figure 26**).

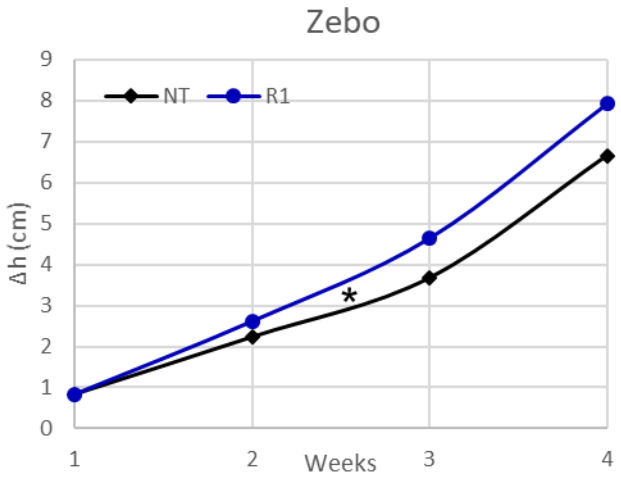


Figure 26 Results of PGP assays carried out with strain R1 on Zebo plants. **A)** graph reporting growth during the experiment. In black is the non-treated control, while in blue is the treated thesis. On the Y axis the Δh is reported, while on the X axis the weeks since the first measurement are reported. Stars indicate significant difference between the treated and non-treated plants throughout the observed period according to One-way ANOVA, optimized for repeated measures (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$)

4.3.1.7 Plant-growth promotion ability of strain R8::*gfp*

Strain R8::*gfp* (*Pantoea agglomerans*) was assayed on several plant species: bean (Nano bobis), zucchini (Genovese), cucumber (Tasty Green), tomato (San Marzano 2) eggplant (Violetta lunga), and pepper (Zebo F1, trial B).

The effect of the inoculation on the different hosts can be summarized in three main categories:

- No effect: the treated tomato, eggplant, and cucumber plants show a slight increase in height, which is not significantly different by the non-treated control (**Figure 21C**, **D**, and **E**);
- Early effect: the treated plants of bean and zucchini showed a statistically significant difference from the non-treated control, but this difference is given by an increased growth in the first weeks, since the final Δh of these plants is the same (**Figure 21A** and **B**);
- Late effect: the treated plants of pepper had the same growth pattern of the non-treated plants at first, but then started growing at an increased rate, determining a statistically significant difference compared to the non-treated control (**Figure 21 F**)

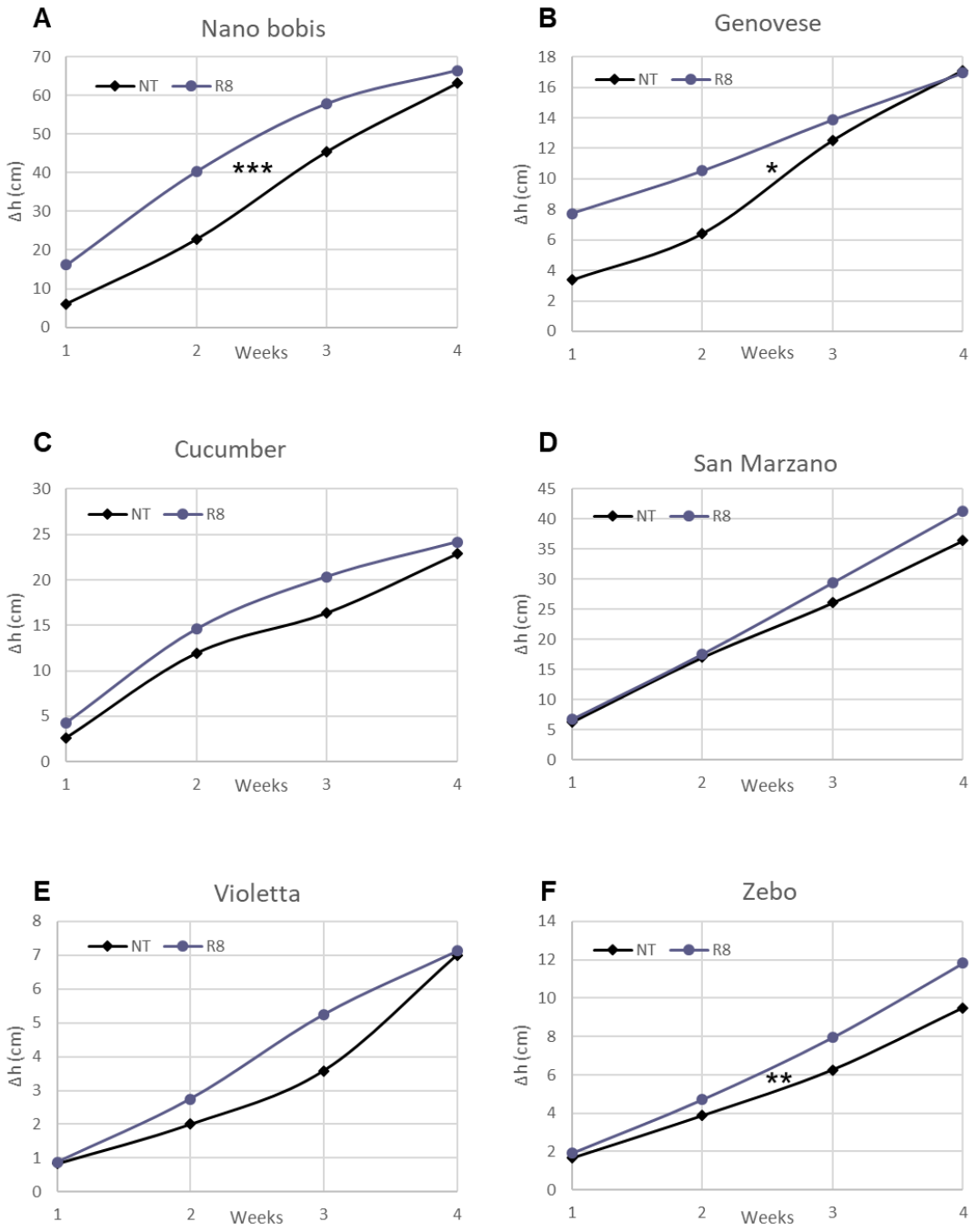


Figure 27 Results of PGP assays carried out with strain R8 on different plant species. In black is the non-treated control, while in indigo is the treated thesis. On the Y axis the difference in height compared to initial measurement of the plant (Δh) is reported, while on the X axis the weeks since the first measurement are reported. Stars indicate significant difference between the treated and non-treated plants throughout the observed period according to One-way ANOVA, optimized for repeated measures (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$)

4.3.1.8 Plant-growth promotion ability of strain R16

Strain R15 (*Paenibacillus pasadenensis*) was assayed on pepper (Zebo, trial B), and there was no detectable difference in height between treated and non-treated control plants throughout the experiment (**Figure 27**).

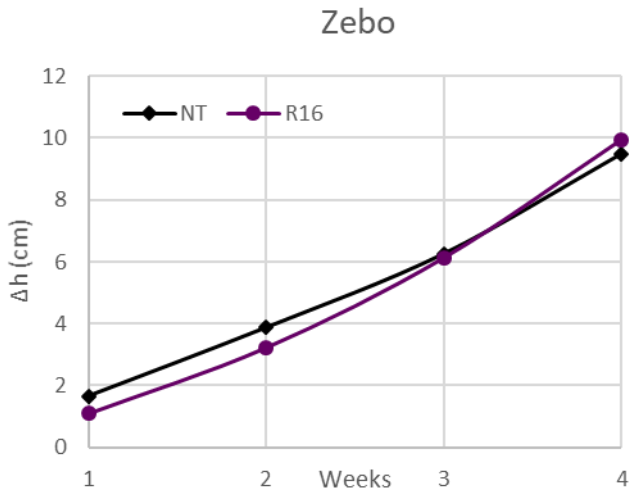


Figure 28 Results of PGP assays carried out with strain R16 on Zebo plants. **A)** graph reporting growth during the experiment. In black is the non-treated control, while in purple is the treated thesis. On the Y axis the Δh is reported, while on the X axis the weeks since the first measurement are reported. Stars indicate significant difference between the treated and non-treated plants according to One-way ANOVA, optimized for repeated measures (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$)

4.3.1.9 Comparison between the PGP ability of the 7 strains

The results obtained on the PGP assay performed on Zebo F1 with the 7 strains were compared, calculating the difference in height as percentage compared to the non-treated control (%h) to eliminate the bias given by the different height of the plants between the three trials (**Figure 29**).

The strain which had the greatest effect in promoting the growth was strain S4C11, followed by strains R8 and 255-7, then strains 255-8, R1, and 260-02, and finally strain R16, which had no growth promotion effect.

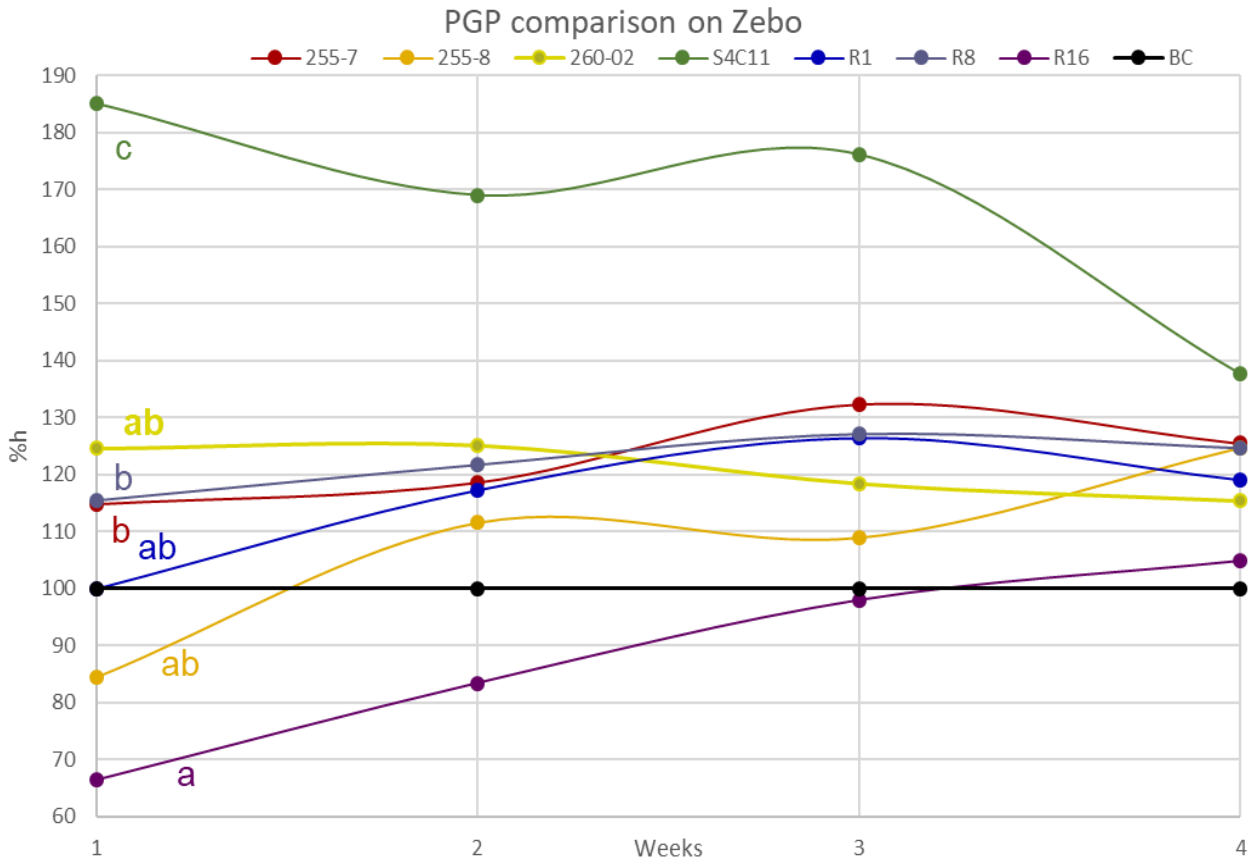


Figure 29 Comparison of the results of PGP assays carried out with the 7 bacterial strains on Zebo plants. The graph reports growth during the experiment. In black is the non-treated control, while in color are the treated theses. On the Y axis the %h is reported, while on the X axis the weeks since the first measurement are reported. Different letters (from a to c) indicate significant statistical difference between the results according to One-way ANOVA, optimized for repeated measures, followed by Tukey's exact post-hoc test ($p < 0.05$).

4.3.1.10 Evaluation of methylation level in Zebo F1 pepper plants

This test, carried out only on the plants used for trial C (non-treated or inoculated with strains 255-7, S4C11, or R1) showed differences in the % of methylated cytosines in the genomes of the plants.

In particular, non-treated plants had higher methylation, ranging from 60% to almost 100%; the plants inoculated with strains S4C11 and R1 had a lower percentage of methylation, ranging from around 50% to almost 80%, although it was not significantly different from the non-treated samples; on the other hand, plants treated with strain 255-7 showed significantly lower levels of methylation, ranging from 30% to 60%, suggesting that several genes are demethylated and activated thanks to the interaction between strain and host (**Figure 30**).

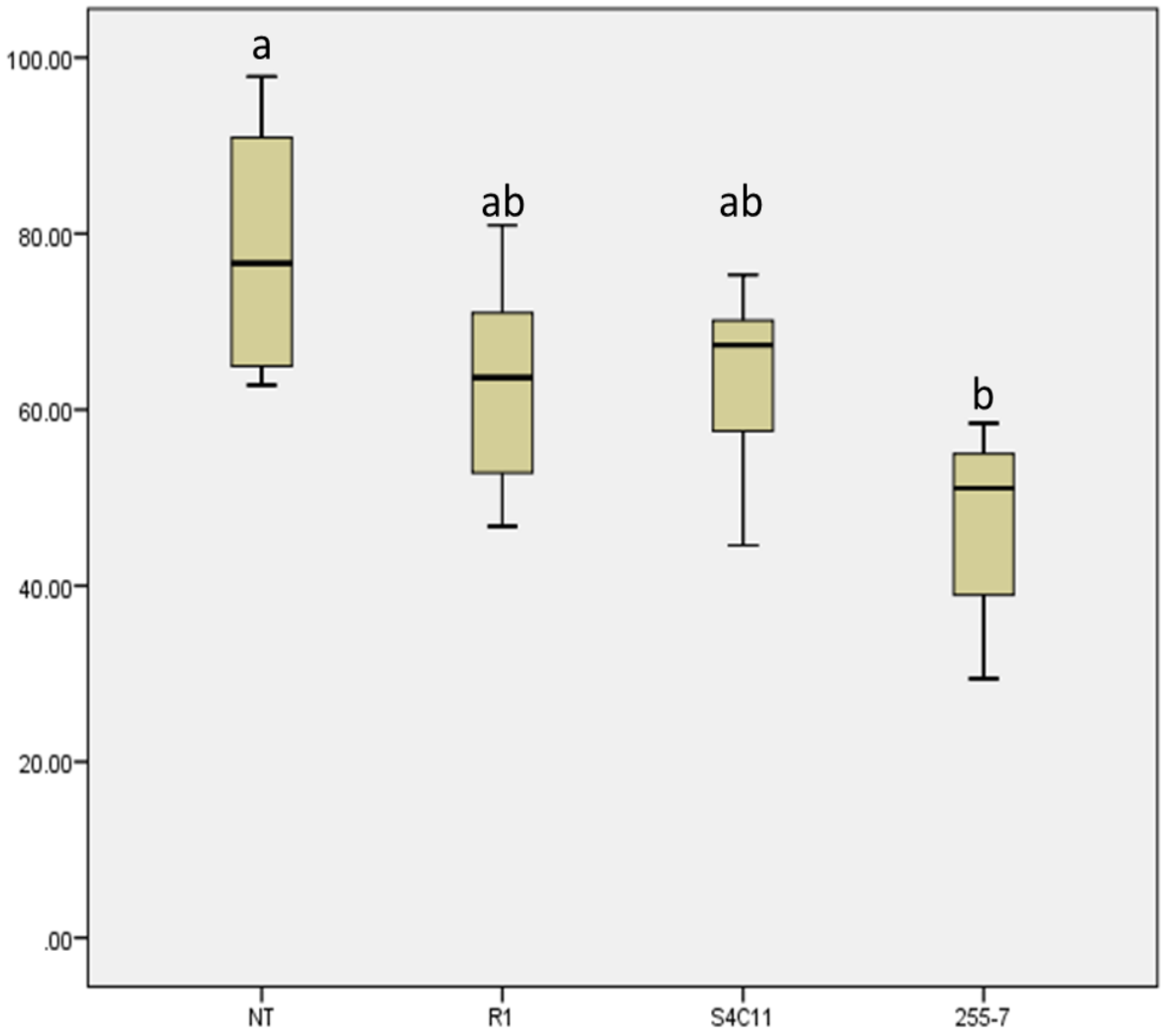


Figure 30 Box-plot describing the result of the assay carried out with 5-mC DNA ELISA kit, reporting the percentage of methylated cytosines detected by the spectrophotometric assay carried out at a wavelength of 450 nm. On the Y axis is reported the percentage of 5-mC (7 plants per treatment), while on the X axis are reported the treatments. Different letters (a,b) identify results which are significantly different according to one-way ANOVA, followed by Tukey's exact post-hoc test ($p < 0.05$).

4.3.2 *In planta* biocontrol assays

4.3.2.1 Biocontrol against *R. solani*

The assays to demonstrate the ability of the bacterial strains to reduce the effects of *R. solani* had inconsistent effects throughout the different trials.

During trial A, involving strain 260-02, the pathogen managed to sensibly reduce both the height and weight of the plants, and induced severe yellowing of the leaves, and there was no significant difference between the plants that were treated only with the pathogen and with both the pathogen and the bacterial strain (**Figure 31C**).

During trials B and C, involving the other 6 strains, the pathogen did reduce the height of the plants, but at the same time significantly increased their weight. While of shorter stature, the infected plants in these two trials did not look unhealthy. Strains 255-7, R1, and R16 had no effect against RS, as the plants treated with the pathogen alone or with both pathogen and bacterial strains have no differences in terms of height or weight (**Figure 31A**, **E**, and **G**). For strains 255-8, S4C11, and R8, instead, there is a different in terms of weight, which is more similar to that of non-treated plants (**Figure 31B**, **D**, and **F**). Still, as this weight is lower than that of the infected plants, it is complex to evaluate this result.

In all the infected plants, PCR assay carried out with the ITS4/ST-RS1 primer pair on DNA extracted from the washed root surface managed to detect the presence of RS, resulting in an amplicon of 187 bp which was not detected in non-infected plants (**Figure 32**), hinting at a successful interaction between pathogen and host, even when the effect on the plants was unorthodox.

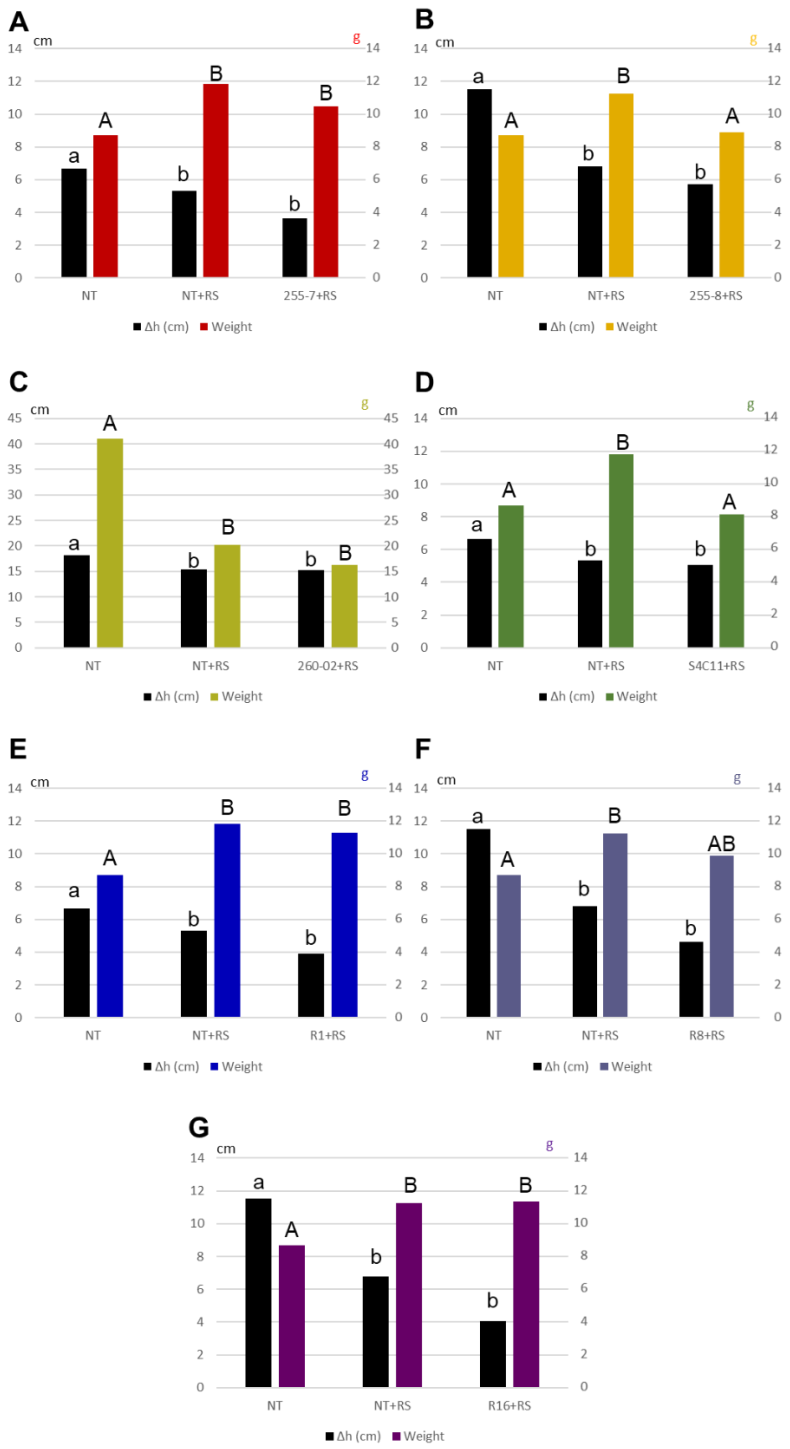


Figure 31 Graphs reporting the results obtained for biocontrol against *Rhizoctonia solani* (RS). In black are reported the average height (cm) of the plants while in color are reported the average weights (g) of the plants (7 plants per treatment). Graphs report the results for different strains, in particular **A)** 255-7; **B)** 255-8; **C)** 260-02; **D)** S4C11; **E)** R1; **F)** R8; **G)** R16. Different lowercase letters (a or b) indicate significant statistical difference between the heights, while different capital letters (A or B) indicate significant statistical difference between the weights, according to One-way ANOVA, followed by Tukey's exact post-hoc test ($p < 0.05$).

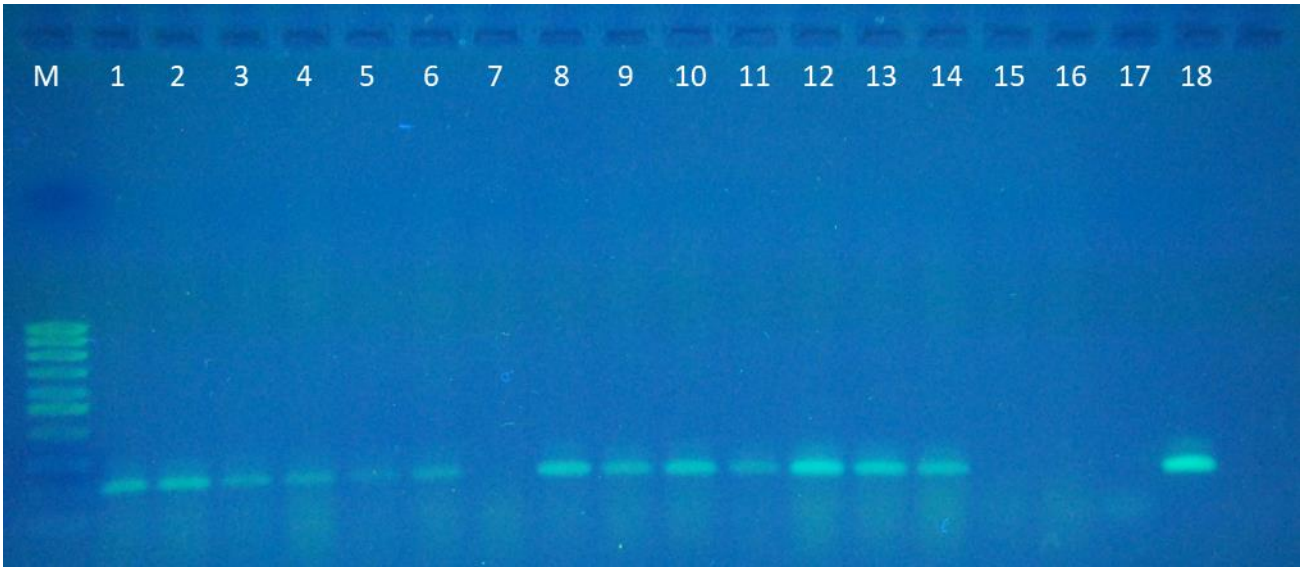


Figure 32 Picture of the agarose gel electrophoresis of PCR carried out to identify the presence of RS in the roots of the samples, using primer pair ITS4/ST-RS1. The different lanes contain: M) ladder SharpMass 100 1-6) non-inoculated pepper plants from experiment B, inoculated with RS; 7) non-inoculated pepper plants from experiment B, non-inoculated with RS; 8-14) pepper plants inoculated with strain 255-8, inoculated with RS; 15) pepper plants inoculated with strain 255-8, non-inoculated with RS; 16-17) negative control (PCR mix with no nucleic acids); 18) positive control (PCR mix with pure RS DNA).

4.3.2.2 Biocontrol against *P. syringae* strains DC3000 and DC3000::*gfp*

The assays to demonstrate the ability of the bacterial strains to reduce the symptoms induced by *P. syringae* strain DC3000 on Zebo plants had positive effects.

The pathogen showed to be able of inducing symptoms on the inoculated plants in all three trials, although during trial A the average number of necrotic lesions per plant was considerably lower (average of 2 lesions per plant) than in the subsequent trials (24-37 lesions per plant).

Strains 255-7 and 260-02 managed to reduce significantly the number of lesions only during the first week, while at the second measurement the number of lesions was no longer significantly different (**Figure 33A** and **C**).

The opposite effect, with a reduction of lesions being visible only during the second week, was observed for strain S4C11 (**Figure 33D**).

All other strains managed to reduce significantly the number of lesions at both measuring times (**Figure 33B**, **E**, **F** and **G**)

The confocal microscopy images obtained from the leaves of non-treated plants without inoculum of DC3000::*gfp*, non-treated plants inoculated with DC3000::*gfp*, and plants that were inoculated at the root with either strain 260-02 or DC3000 and on the leaves with DC3000::*gfp* offer further evidence of the effect of the strain in controlling the colonization by the pathogenic *P. syringae* (**Figure 34**), and how the inoculum of a different, pathogenic strain can instead affect negatively the host's defense against the pathogen. It is noticeable how in the plants that were treated with 260-02 less fluorescent cells are detectable on the surface of the leaves, both after 3 and 6 days from the inoculum of the pathogen. A much more consistent colonization by strain DC3000::*gfp* can instead be noticed in plants treated at the root with strain DC3000.

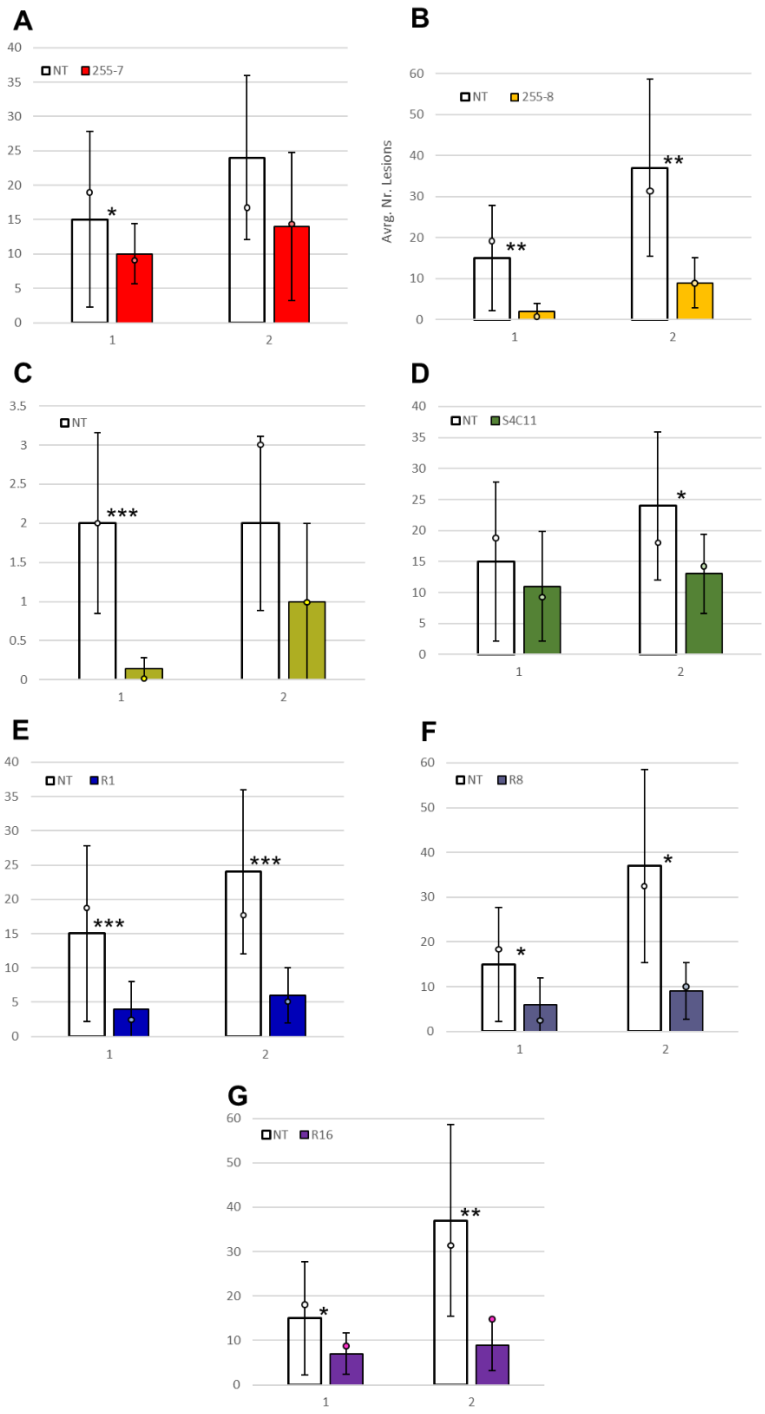


Figure 33 Graphs reporting the results obtained for biocontrol against *P. syringae* strain DC3000. In white are reported the average number of lesions for non-treated control plants, and in color the one registered for the bacterial strains (7 plants per treatment). Error bars indicate standard deviation, while the dots indicate the median value. Graphs report the results for different strains, in particular **A)** 255-7; **B)** 255-8; **C)** 260-02; **D)** S4C11; **E)** R1; **F)** R8; **G)** R16. Stars indicate significant difference between the treated and non-treated plants according to Mann-Whitney U-test (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$)

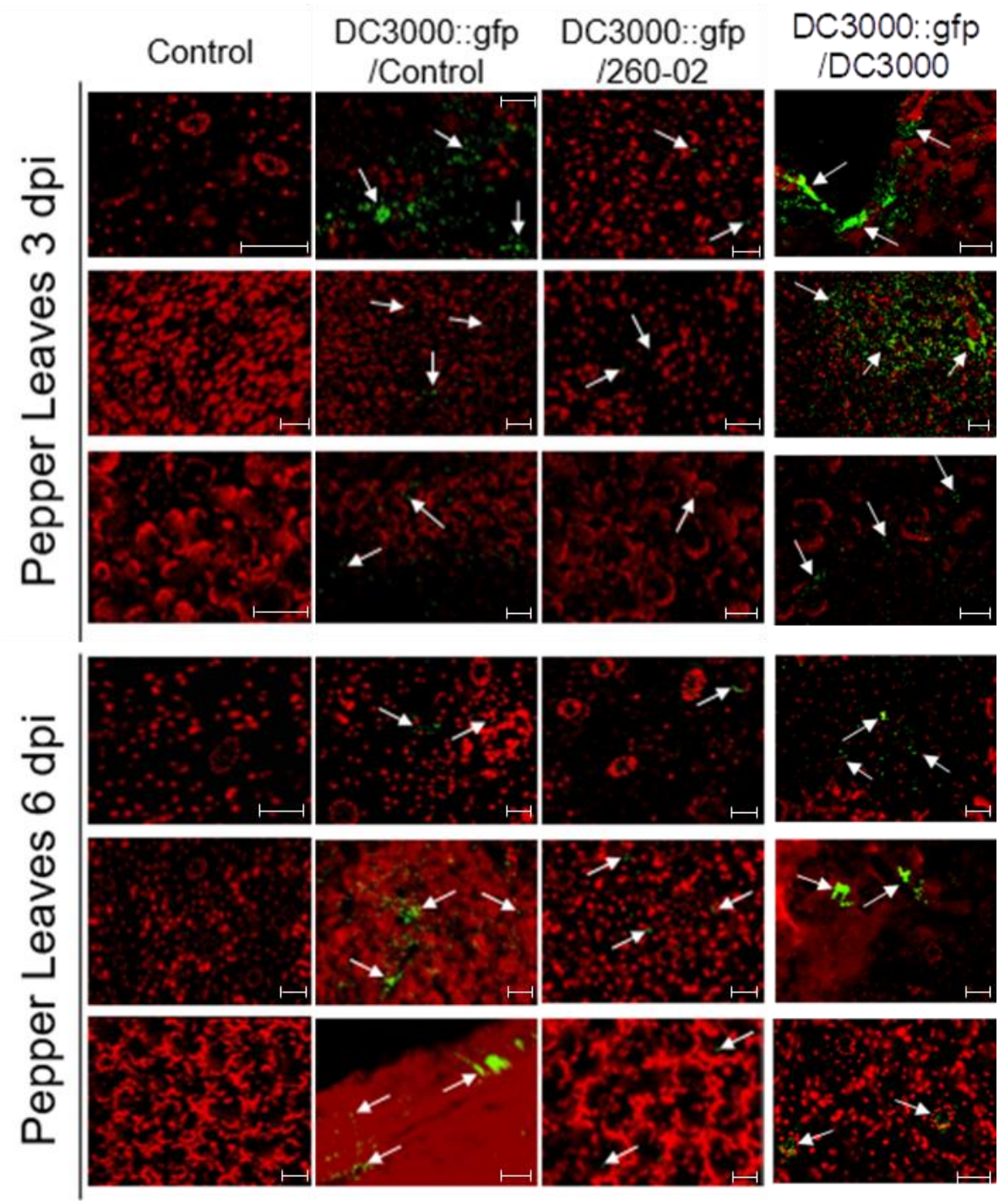


Figure 34 Panel of photographs taken by laser confocal microscopy: red indicates the structure of plant tissues, while green indicates cells of *P. syringae* strain DC3000::*gfp*. White arrows highlight zones where green cells can be seen. Control pictures are shown to prove that no endogenous green fluorescence could be detected, and that the fluorescent cells come from the spraying of the leaves with strain DC3000::*gfp*. Images are divided in two groups by the time after which they were taken (3 or 6 days) and compared between control, inoculated only with DC3000::*gfp*, inoculated with DC3000::*gfp* on the leaves and strain 260-02 at the root, or inoculated with strain DC3000::*gfp* on the leaves and strain DC3000 at the root. Scale bars in each picture are 15 µm in length.

4.3.2.4 Biocontrol against *Cymbidium Ringspot Virus*

The assays to evaluate the ability of the 7 bacterial strains to reduce the incidence of CymRSV had variable effects, depending on the strains.

The pathogen managed to develop very evident symptoms on Zebo plants, causing necrotic spots on the inoculated leaves as well as spreading systemically to non-inoculated leaves. In a few cases, the largest spots developed into the ring pattern that the virus is named after (**Figure 35A**).

During trial B, the virus infection was particularly severe on the plants, causing crippling symptoms on the plants (**Figure 35B**), which died soon thereafter, making a quantification of the symptoms impossible. Since out of the plants treated with the strains included in trial B (255-8, R8, and R16) only those treated with strain R16 could survive and kept a mostly healthy appearance (**Figure 35C**), it can be concluded that strain R16 exerted an effect against the disease, while strains 255-8 and R8 did not.

During trials A and C, instead, the virus developed non-lethal symptoms on the plants, which made the examination and attribution of severity possible. Strains R1 and S4C11 had no effect against the virus infection, and the symptoms displayed are the same as those in non-treated plants (**Figure 36C** and **D**), while strains 260-02 and 255-7 managed to reduce significantly the severity of the symptoms on the plants inoculated with those strains (**Figure 36A** and **B**).

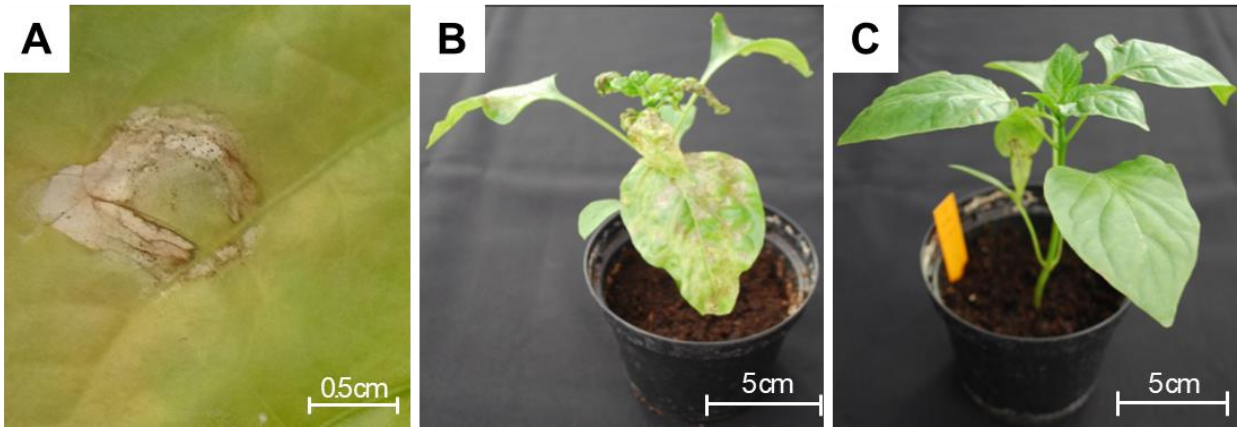


Figure 35 Photographs displaying the phenotypic effect of CymRSV on Zebo plants. **A)** typical necrotic symptom, developing into a ring shape; **B)** non-treated control plant, inoculated only with CymRSV, during trial B, displaying severe symptoms; **C)** plant treated with strain R16 before being inoculated with the virus, showing only mild symptoms.

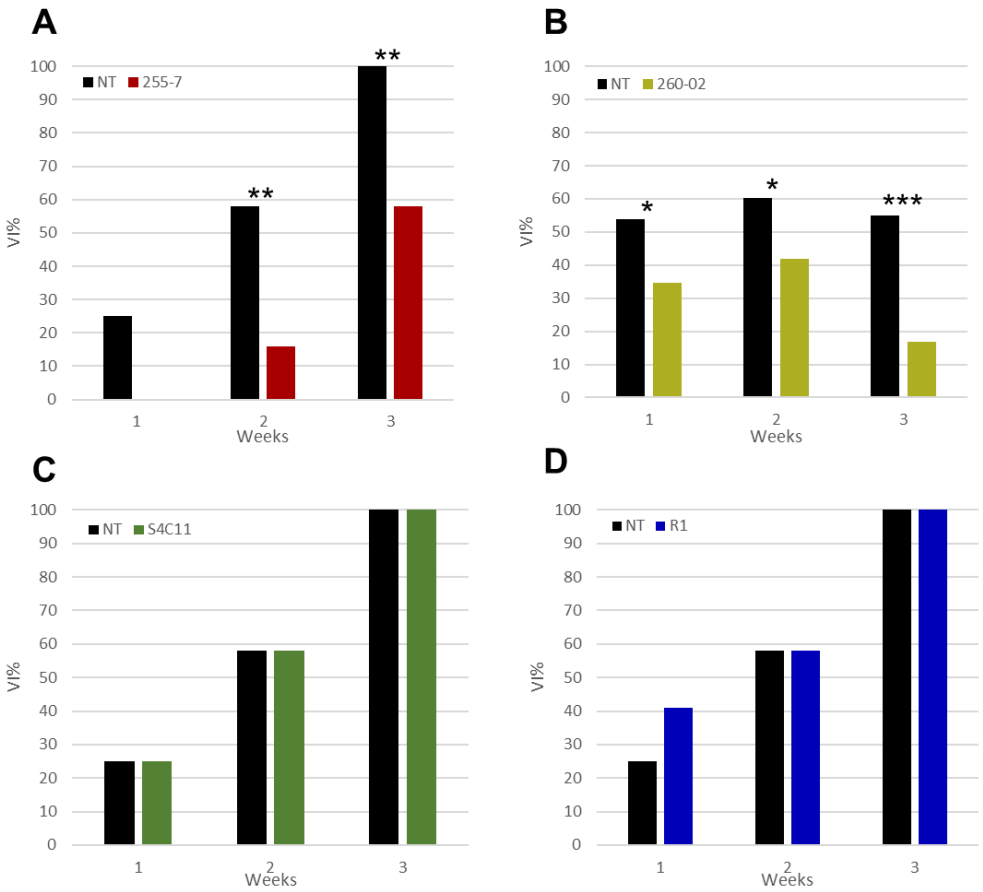


Figure 36 Graphs reporting the results obtained for biocontrol against CymRSV. In black are reported the percentage of virus infection (VI%) for non-treated control plants, and in color the one registered for the bacterial strains. Graphs report the results for different strains, in particular **A)** 255-7; **B)** 260-02; **C)** S4C11; **D)** R1. Stars indicate significant difference between the treated and non-treated plants according to Mann-Whitney U-test (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$)

Along with the phenotypic observation of the virus symptoms, gene expression assays were carried out to quantify both the quantity of virus and of some plant genes involved in the resistance against pathogens: phenylalanine ammonia lyase (PAL) and pathogenesis-related protein 1 (PR1-a).

At the first measurement (1 day after infection), PAL gene expression was highest in the non-treated plants, showing that the presence of the virus was downregulating the gene, regardless of the presence of the bacteria, with the exception of strain R1, in which the gene was even more downregulated, possibly explaining at least in part why these plants displayed severe symptoms (**Figure 37A**). At the second measurement (14 days after infection), there was a general increase in the expression level of this gene, in particular regarding strains 255-8 and R8, which were probably trying to compensate for the severe symptoms they were already displaying, since instead the more healthy-looking R16-treated plants did not show this large increase in the expression of this gene (**Figure 37B**).

Gene PR1-a showed no difference between the treatments during the first measurement (**Figure 37C**). Instead, it shows a high upregulation in samples treated with strain 255-7 and S4C11 during the second measuring (**Figure 37D**), even though the two have very different phenotypes.

Regarding the concentration of virus, in both measurements the concentration was much higher than in all other samples in the plants treated with strain 255-7, an unexpected result considering the low level of symptoms seen in those plants (**Figure 37E** and **F**).

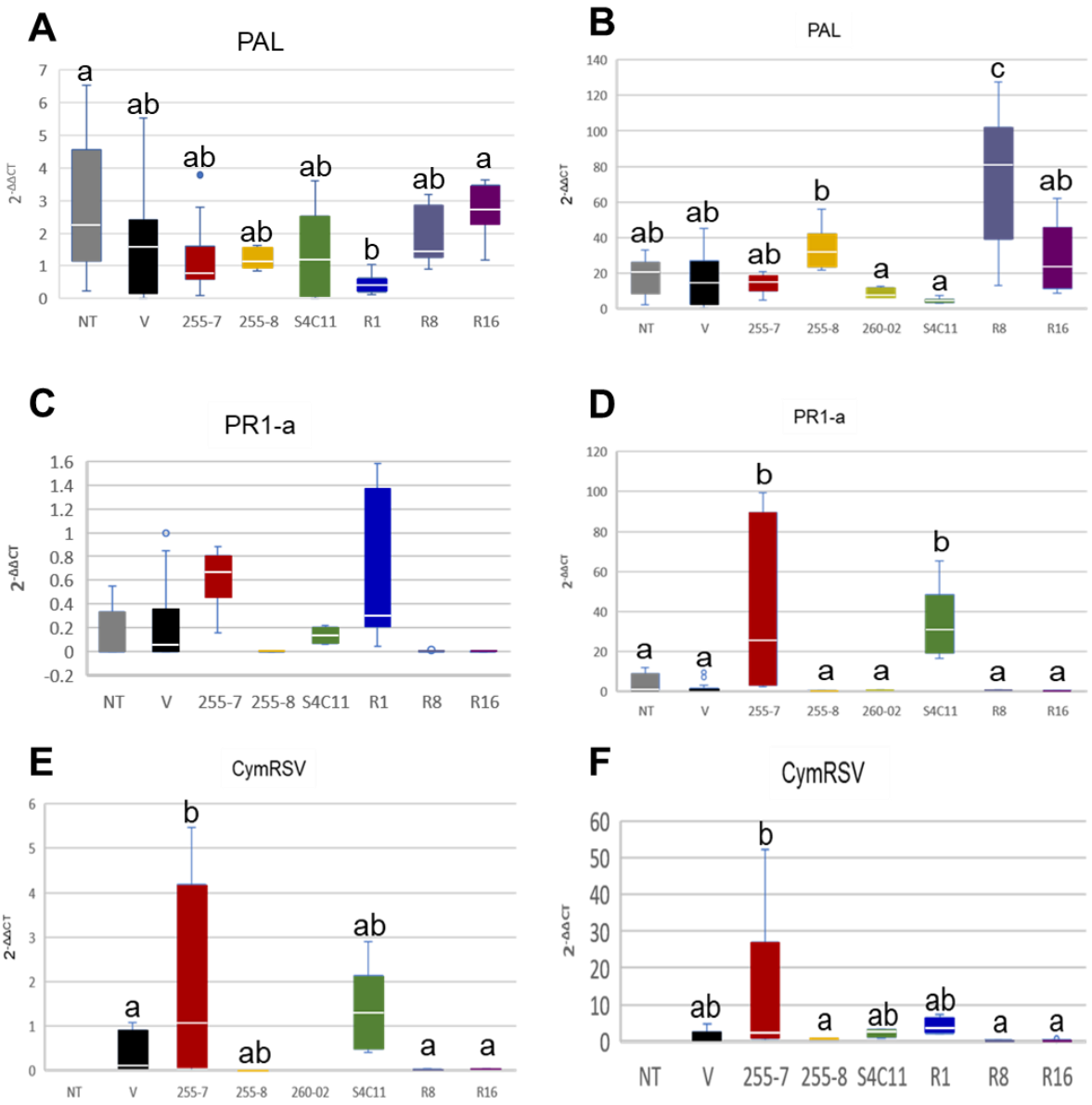


Figure 37 Results regarding the relative gene expression assays in Zebo plants, either non-treated, inoculated with CymRSV alone, or inoculated with both CymRSV and one of the bacterial strains. On the Y axis is reported the $2^{-\Delta\Delta CT}$ value of each gene, normalized on actin, and on the X axis are reported the different treatments. A) Relative expression of PAL gene at first measurement; B) Relative expression of PAL gene at second measurement; C) Relative expression of PR1-a gene at first measurement; D) Relative expression of PR1-a gene at second measurement; E) Relative quantity of CymRSV at first measurement; F) Relative expression of CymRSV at second measurement. Different letters (a or b) indicate significant statistical difference between the results according to One-way ANOVA, followed by Tukey's exact post-hoc test ($p < 0.05$).

4.4 Results of *in silico* analyses

In this chapter are reported all the results obtained from analyses carried out on the genome of the 7 bacterial strains.

4.4.1 Genome assembly

The statistics of the assembly of the reads obtained through Illumina sequencing for all 7 strains, and through hybrid approach between Illumina and MinION technologies for strain R16, are reported in **Table 8**.

All genomes assembled from Illumina reads, with the exception of that of strain 255-8, had satisfactory statistics. The genomes showed a variable degree of fragmentation, ranging between 35 contigs for the genome of strain R1 to 190 of strain R8, generally showing a higher degree of fragmentation as the total length of the genome increases. These genomes had a total length comparable to that of other genomes already deposited in NCBI for other bacteria of the same species or genus, confirming the assembly data.

The exception was strain 255-8, which produced a highly fragmented genome with a disproportionately large total size. Extracting the 16S gene from this genome provided two distinct sequences, one of which belonged to *Burkholderia* sp. Strain 255-8, and the other one had a high similarity to sequences of *Staphylococcus* spp.

Considering that also the abnormally high genome size could be explained by the assembly containing both the large genome of *Burkholderia* and the smaller genome of *Staphylococcus*, the conclusion was that there was a contamination in the preparation of the sample for sequencing.

The comparison between the genome obtained for strain R16 with the two different techniques gives a clear indication on the added value brought by the long reads generated with MinION sequencing. The two assemblies show the same level of GC content and almost the same total size, indicating that the accuracy of Illumina sequencing was not lost, but the overall quality of the assembly is without doubt much higher, reducing the fragmentation of the genomic data.

Due to its higher quality, the assembly obtained with this hybrid approach was used for all subsequent analyses on strain R16.

Table 8 Table resuming the assembly statistics of the genomes for the 7 bacterial strains. Total number of scaffolds, maximum length of these scaffolds, total genome size, and percentage of GC content in the genome are reported for each assembly. All the assemblies, except for column R16 Hyb, were obtained from Illumina reads only. The column R16 Hyb is the assembly performed with hybrid technique using data from both Illumina and MinION sequencing technologies.

	255-7	255-8	260-02	S4C11	R1	R8	R16	R16 Hyb
Scaffold Number	36	751	43	55	35	190	96	**5**
Max Length (MBp)	2.253	0.528	0.909	1.363	0.974	0.787	0.643	**3.401**
Genome size (MBp)	4.753	12.385	6.052	5.007	3.728	9.172	5.727	**5.753**
GC% content	55	54	59	36	41	61	63	**63**

4.4.2 Genome annotation

The assembled genomes were annotated automatically through the RAST software, which detects coding sequences in the genome and assigns a putative function to them by comparison with an internal database.

The characterized sequences are then inserted in “subsystems”, which describe metabolic pathways or other closely related functions, offering a more complete description of the biological functions associated to the analyzed genomes.

According to these two criteria, each coding sequence can be characterized either as hypothetical (H) or non-hypothetical (NH), and as being part of a subsystem (SS) or not part of any subsystems (NSS).

The result of the RAST annotation for the 6 assembled genomes is reported in **Table 9**.

Table 9 Table reporting the results obtained from the annotation of the assembled genomes through RAST. Data reported includes total number of coding sequences (N° CDS), number of coding sequences per Kbp of genome (CDS/KBp), number of sequences that are non-hypothetical and assigned to a subsystem (NH – SS), number of sequences that are hypothetical and assigned to a subsystem (H – SS), number of sequences that are non-hypothetical and not assigned to a subsystem (NH – NSS), and sequences that are hypothetical and not assigned to a subsystem (H – NSS).

	255-7	260-02	S4C11	R1	R8	R16
N° CDS	4385	5235	5033	3860	8499	4881
CDS/KBp	0.92	0.86	1.00	1.03	0.92	0.85
NH – SS	2270	2513	2022	1769	3512	1962
H – SS	144	146	126	100	226	86
NH – NSS	1131	1530	1262	909	2476	1419
H – NSS	840	1046	1623	1082	2285	1524

4.4.3 Functional genome analysis

Genes that could be related to traits associated to rhizosphere competence, endophytic lifestyle, plant-growth promotion, and biocontrol were searched in the annotated genomes during a preliminary functional analysis.

The categories that were investigated included predominantly: secretion systems, motility and chemotaxis, secondary metabolites, detoxification pathways, and some specific pathways of carbon utilization (fructose and chitin).

4.4.3.1 Secretion systems

Investigating the secretion systems included in the genome is of great importance since many of these systems, in particular Type III, IV, and VI, are known to be involved in the interaction with the host and can affect colonization success of pathogenicity/plant-growth promoting effect of the microorganism (Mitter *et al.*, 2013).

Since all these secretion systems are typical of Gram-negative bacteria, the result of finding no genes related to these systems in Gram-positive strains S4C11 and R1 is expected, while it is interesting to find genes related to systems II and IV in strain R16, likewise Gram-positive (**Table 7**).

For the Gram-negative strains, it is possible to see that all the strains' genomes encoded for Type II, IV, and V secretion systems, Type VI is present only in strains 255-7 and 260-02, while type III is present only in strain 260-02, and Type VII is present only in strain 255-7 (**Table 10**).

No strain presented genes typical of Type I secretion system.

Table 10 Table reporting the number of genes related to secretion systems from I to VII in the 6 genomes of the bacterial strains.

	255-7	260-02	S4C11	R1	R8	R16
Type I SS	0	0	0	0	0	0
Type II SS	6	17	0	0	34	12
Type III SS	0	30	0	0	0	0
Type IV SS	12	23	0	0	50	10
Type V SS	6	6	0	0	6	0
Type VI SS	26	30	0	0	0	0
Type VII SS	5	1	0	0	0	0

4.4.3.2 Motility and chemotaxis

The ability of a microorganism to move (motility) and to direct its movement towards the source of a chemical stimulus (chemotaxis) are very important traits for rhizosphere competence, and can help determining whether a candidate biocontrol agent can be successfully employed in field or not.

All 6 genomes encoded a large number of genes related to the production and utilization of flagella for movement as well as chemotaxis, with strain R8 possessing the highest number for both categories (**Table 11**).

It is also worth mentioning in this paragraph that the pili produced as part of Type IV secretion system, discussed in the previous paragraph, are involved in a different motility mechanism described as writhing motility, which is often important in soil (Berry and Pelicic, 2015).

Table 11 Table reported the number of genes related to the production and utilization of flagella, and chemotaxis in the 6 genomes of the bacterial strains.

	255-7	260-02	S4C11	R1	R8	R16
Motility	74	89	82	71	96	21
Chemotaxis	49	54	54	15	56	20

4.4.3.3 Secondary metabolites

Every one of the considered strains encoded for the production of quite the diverse quality of secondary metabolites. In this preliminary analysis, the main focus was put on the following categories:

- Siderophore and indole-acetic acid production, to confirm the results obtained from the biochemical assays
- Production of terpenes, toxins, and ability to carry out butanol fermentation, to confirm the results obtained from the competition assays with fungi

All the 6 genomes encoded for genes related to auxin production, in line with the results obtained *in vitro*, while siderophore production was confirmed as being present for strains 255-7, 260-02, S4C11, and R1, confirmed as being absent for strain R8 but, surprisingly, a large number of genes related to the utilization of siderophores was identified in strain R16 (**Table 12**). Among these genes, though, there seemed to be no full pathway for the production of siderophores, confirming that the strain indeed does not produce them.

All 6 bacterial genomes possessed genes for the butanol fermentation pathway, albeit with a variable quantity of genes, with strain 260-02 having less than others, and strain 255-7 having more. A similar situation was detected with toxin production, with all the strains being able to encode for the production of at least some bacteriocins, and with strain 260-02 having the most diverse array of toxin-related genes (**Table 12**).

Confirming the volatile compounds profile, only strain R16 showed a large quantity of genes related to terpene production, showing an almost complete pathway for production of farnesol, while the other strains had a smaller quantity of genes related to terpenes (**Table 12**).

Table 12 Table reporting the results of the functional analysis on the 6 bacterial genomes. For genes related to siderophore production and utilization, and Indole-acetic acid (IAA) production, the number of genes per strain is reported. For genes related to fermentation of butanol, production of toxins, and production of terpenes, the results are expressed with a number of + symbols expressing the abundance, with + being the lowest and +++ being the highest number of genes.

	255-7	260-02	S4C11	R1	R8	R16
Siderophores	19	18	36	23	3	24
IAA	4	4	5	4	5	7
Fermentation	+++	+	++	++	++	++
Toxins	+	+++	+	++	++	+
Terpenes	+	+	+	+	+	+++

4.4.3.4 Detoxification and substrate use

In this preliminary analysis, the genes related to the detoxification of reactive oxygen species (ROS) were researched, as production of ROS is a widespread phenomenon inside plant tissues, both as part of regular metabolism and as response to stresses, and the ability to detoxify these compounds is important for endophytes to survive inside the plant (Mitter *et al.*, 2013).

All the genomes had several genes encoding for proteins that detoxify ROS, always including at least one catalase and superoxide dismutase, with a variable total number of genes ranging between 38 for strain S4C11 and 108 for strain R8 (**Table 13**). It is noticeable a clear difference in the number of these genes among the three Gram-positive strains (S4C11, R1, R16) and the Gram-negative ones (255-7, 260-02, R8), with the latter having around twice the amount of these genes compared to the former.

This analysis focused on two specific substrates: fructose, which is highly abundant inside plant tissues and can be a marker for adaptation to endophytic lifestyle, and chitin, which is the main component of fungal cell wall and can indicate some degree of direct parasitism towards phythopathogenic fungi.

All 6 strains showed genes related to the utilization of fructose, indicating that these strains are capable of using fructose as an efficient carbon source for glycolysis (**Table 13**).

On the other hand, only strain R16 demonstrated a full pathway for utilization of chitin as a carbon and nitrogen source; this result is in line with the biochemical assays, which showed that only this strain had a chitinolytic activity *in vitro* (**Table 13**).

Table 13 Table reporting the number of genes related to detoxification of reactive oxygen species (ROS), utilization of fructose as a carbon source, and utilization of chitin as a carbon and nitrogen source.

	255-7	260-02	S4C11	R1	R8	R16
ROS detox	73	81	38	39	108	41
Fructose	11	8	7	24	9	37
Chitin	10	0	14	6	6	71

4.4.4 Comparative pangenome analysis

The unique characteristics among known *P. syringae* strains of strain 260-02, and the wide availability of several fully-sequenced genomes for this species, brought this study to focus on comparative genomic analysis for this strain.

The pangenome analysis carried out in this study compared the gene content of 9 genomes from pathogenic *P. syringae* strains, 1 genome from pathogenic *P. savastanoi* strain, and the genome of strain 260-02 with the aim of characterizing which genes differentiate the pathogenic strains from the beneficial one.

The output of the pangenome analysis was manually analyzed to detect the genes that were present in pathogenic strains and not in strain 260-02, and the genes that differentiated strain 260-02 from its closest relative, strain UMAF0158, which showed a similarity of approximately 99% with strain 260-02 and has a pathogenic behavior. Furthermore, the genes that were reported as being present exclusively in 260-02 and not in the pathogenic strains were searched.

Not giving any information apart from presence or absence, all genes that were identified only as "Hypothetical protein" were ignored for the present study. These genes could possibly be very important for pathogenicity, but at the moment they cannot be analyzed through bioinformatics without a proper characterization.

In total, the genes encoding non-hypothetical proteins identified as being different between strain 260-02 and the analyzed pathogenic strains are 117, and they were grouped according to their function or pathway (**Table 14**), with the category labelled "Other" containing the few genes that had no similarity to other genes and being reported as coding for: acetoin catabolism regulatory protein, benzaldehyde dehydrogenase, and two different pesticin receptor precursors.

The highest number of different genes is in the category that includes toxins or effectors, with 40 genes that were present in the various strains and were not identified in strain 260-02 (**Table 14**). Two of these genes were identified also in strain UMAF0158, and could therefore be important determinants for pathogenic behavior: (i) ornithine aminotransferase, which could be related to the production of typical *P. syringae* toxins that interfere with the synthesis of arginine by inhibiting an enzyme that works on the same substrate (ornithine N-acyl transferase) (Carrion *et al.*, 2012); (ii) Virulence regulon transcription activator VirF, which gets its name from the

system identified in animal pathogens, such as *Shigella flexneri,* and is crucial to host invasion (Dorman *et al.*, 2001), but could be related to expression of virulence also in *P. syringae*.

Another function in which several genes are different is the quorum sensing category, with 18 genes missing from 260-02, of which 2 are present in its closest relative (**Table 14**). These genes are identified as (i) a specific LuxR receptor. Strain 260-02 presents several LuxR genes, but does not show this specific one. Although LuxR genes are traditionally associated with sensing acyl-homoserine lactones (AHL) and mediating the response to quorum sensing, there are several genes of the LuxR family that bind other molecules (Patel *et al.*, 2013) it will be therefore be interesting to characterize the specific substrate of this LuxR gene. And (ii) an homoserine/homoserine lactone efflux pump, which could mediate uptake of AHL and determine quorum sensing efficiency. Another gene of note not present in strain 260-02, although missing also in UMAF0158, is PsyI, the homologue of LuxI, responsible for the synthesis of AHL (Antunes and Ferreira, 2009), which hints at the possibility that strain 260-02 can perceive quorum sensing molecules but does not directly contribute to achieving the quorum threshold.

There are several different genes identified in the secretion systems, with 15 genes for Type II, 14 for Type III, 17 for Type IV, and 1 for Type VI (**Table 14**). Although the most well-known pathogenesis-related secretion system for *P. syringae* is Type III (Mudgett, 2005; Tang *et al.*, 2006; Lindeberg *et al.*, 2012), the fact that none of the 14 genes identified as missing from 260-02 are present in strain UMAF0158 could mean that none of those genes are necessary for the pathogenicity. Instead, 7 among the 17 genes related to Type IV secretion system which are missing in 260-02 are present in UMAF0158, indicating that these genes could have a direct impact on pathogenicity. These genes mostly encode for different VirB proteins, which are important determinants for the virulence in *Agrobacterium tumefaciens* (Berger and Christie, 1994), but such a role is unknown for *P. syringae*.

The category indicated as gene expression contains transcription activators and methylation proteins, for a total of 8 genes (**Table 14**). Of these genes, only one in present in UMAF0158 and not in 260-02, a DpnIIA modification methylase.

Of the 4 genes in the "Other" category, none is present in UMAF0158, and they are therefore unlikely to be important determinants of pathogenicity.

Only 8 genes were present exclusively in strain 260-02 and in no other *P. syringae* genome included in the analysis.

Of these genes, 4 are defense genes: 3 are related to arsenate resistance, and one protects the bacterium against the bacteriocin colicin.

One gene is a chitinase, which does not seem to be active in the genome, considering that the strain showed no chitinolytic activity *in vitro*.

The last three genes are the ones that seemed most interesting, being three DpnIIA modification methylases which are different from other proteins in this class present in other *P. syringae* genome.

The DpnIIA gene 1238 has a high similarity with a methylase present in *P. syringae* pv. *japonica* (90% cover, 98% identity on the AA sequence), but finds no other match in the *Pseudomonas* genus and, interestingly, a BLAST analysis of the nucleotide sequence gives only one match (cover 9%, identity 76%) with the genome of *Desulfotomaculum reducens* strain MI-1. Utilizing the discontiguous megablast algorithm instead of the regular megablast one, there is a single match within the *Pseudomonas* genus on the genome of *P. psychrotolerans* strain PRS08-11306 (coverage 93%, identity 72%).

The DpnIIA gene 1735 shares only 80% of similarity at AA level, and the least similar one not even finding any hit on other *P. syringae* genomes by BLAST.

The DpnIIA gene 4148 gene shares 95% of similarity at AA level with a single *P. syringae* strain (identified as Leaf127), but has no other hit on the *Pseudomonas* genus, with the next most similar protein sharing 79% of identity and being identified in *Burkholderia* sp. Strain CF099.

Table 14 Table reporting the number of genes not present in the genome of strain 260-02 but found in the other, pathogenic strains included in the pangenome analysis. The right column reports how many of these genes are detected in the closest relative to strain 260-02, strain UMAF0158.

	Pangenome strains	*P. syringae* UMAF0158
Toxin/Effectors	40	2
Quorum sensing	18	2
SS Type II	15	0
SS Type III	14	0
SS Type IV	17	7
SS Type VI	1	0
Gene expression	8	1
Other	4	0

5 Discussion

In the context of modern agriculture, sustainability and food safety and security are very important topics (Carvalho, 2006; Pickett, 2013; Bajwa, 2014; Curtis and Halford, 2014;). Plant diseases are a threat and challenge towards both of these themes: much of the pollution and threat to environment from agriculture comes from the use of pesticides employed to control diseases and pests; at the same time, plant diseases can severely impair yield or quality of crops if left unchecked, but food safety is also threatened by the use of pesticides in agriculture (Carvalho 2006; Damalas and Eleftherohorinos, 2011).

In this complex scenario, alternative ways of controlling plant diseases in a more sustainable way are a needed implementation in agricultural practices. A promising method among these alternative control strategies can be found in biological control (Johnson *et al.*, 2010).

The isolation of effective biocontrol agents can be complex since there are no clear markers to infer whether a microorganism is capable of having a positive effect on a plant host without actually testing the effect of the microorganism. There are still some guidelines, such as selecting microorganisms that were isolated from plant tissues or soil, indicating their ability to survive in the right environment, and using previous literature as guidelines, since some genera are more known for their plant-growth promoting and/or biocontrol ability.

Many recent studies describe how diseases can restructure the microbiota of a host plant and, observing the same data with the opposite goal, try to describe the structure of a "healthy microbiota" for that crop plant, with the final aim of working on the microbial community inside the plant to affect its sanitary status, preventing the pathogen attack or inducing recovery from diseases (Trivedi *et al.*, 2010, Bulgari *et al.*, 2011, Podolich *et al.*, 2015, Larousse *et al.*, 2017).

With these information as a starting point, this study investigated bacteria isolated from two separate sources: (i) grapevine plants of the Barbera variety that suffered from flavescence dorée phytoplasmatic disease and underwent a natural recovery process (Bulgari *et al.*, 2011), or (ii) apple plants that were not suffering from apple proliferation phytoplasmatic disease in orchards that were characterized by a high incidence of the disease (Spreafico, 2012-2013).

Both of these starting materials offered an interesting opportunity to identify bacterial biocontrol agents since the host plants showed a particular behavior regarding these phytoplasmatic diseases.

In the present doctoral thesis, work was carried out on selected bacterial strains from the collection present in the Biodefense unit of DISAA, which had already been identified as strains putatively related to biocontrol from previous studies (Bulgari *et al.*, 2011; Spreafico, 2012-2013), both identifying biochemical features related to biocontrol or plant-growth promoting traits, and in microbiome analysis.

Furthermore, these strains were previously characterized through *in vitro* biochemical assays that revealed several traits associated to plant-growth promoting rhizobacteria (PGPR), rhizosphere competence and endophytic lifestyle.

The hypothesis at the basis of this study is that the sanitary status of the host plants could have been influenced, at least in part, by the presence of these bacterial strains in the host plant.

This study proceeded to assay these bacteria in a three-step approach, starting from *in vitro* preliminary assays, moving to *in vivo* assays, and concluding with *in planta* assays to determine whether the selected strains could promote plant growth and, more importantly, control diseases caused by bacterial, fungal, and viral pathogens.

In general, the *in vitro* dual-culture inhibition assays proved to be effective for all strains against 4 of the 5 pathogens considered in the study (*Aspergillus* sez. *nigri* strain As-N1, *Botrytis cinerea* strain MG53, *Phomopsis viticola* strain PV1, and *Rhizoctonia solani* strain RS1) with an inhibition rate that could range between 47% and 100% for different pathogen-bacteria combinations. No relevant effect was identified for the last pathogen (*Fusarium verticillioides* strain GV2245) (**Figures 3 to 7**). Our strains had no effect on *F. verticillioides*, but there are several strains of fungi or bacteria reported in literature to control the growth of all the studied phytopathogenic fungi *in vitro*, including *F. verticillioides* (Ligon *et al.*, 2000; Tourè *et al.*, 2003; Siddiqui *et al.*, 2004; Cavaglieri *et al.*, 2005, Nayaka *et al.*, 2009; Dalie *et al.*, 2010; Kotze *et al.*, 2011; Ji *et al.*, 2013; Abiala *et al.*, 2015). There is also to note that one of the strains (R16) demonstrated a slight *in vitro* inhibitory effect towards the growth of FV in different experimental conditions (Passera *et al.*, 2017); in this assay, the strain was streaked in half of a petri dish, and in the middle of the other half the fungus was inoculated. Seeing how this setup gave some results, it may be possible that the fungal strains requires a much higher number of bacterial cells, and the molecules they produce, to be inhibited, or that only direct contact with the bacterial cells enables the effect.

The *in vitro* dual-plate inhibition assays were then carried out in order to prove if the strains could inhibit myceliar growth of some of these fungi (BC, FV, and PV) through volatile molecules alone. Once again, no relevant effect was detected for FV, while the growth of BC and PV could be inhibited by the strains in these conditions (**Figures 9 and 10**). The inhibition obtained in the dual-plate assay proves the production of volatile molecules, while the lower inhibition values registered in this assay suggests the presence also of other active molecules which are not volatile

These results indicate that the strains are capable of producing molecules that can directly inhibit the growth of a wide range of fungal pathogens, belonging to different phyla (*Ascomycota, Basidiomycota*) but showing a certain specificity, since not all pathogens were affected with the same efficiency, and one of them was completely unaffected.

Also, the bacteria showed an immediate inhibitory effect against the fungus (Penaca, 2015-2016; Passera *et al.*, 2017) and this effect was long-lasting, being maintained for at least 14 days, which is a longer period of time than what is generally evaluated in this kind of assay, being 5 or 7 days (Essghaier *et al.*, 2009; Todorova and Zozhuharova, 2009).

After proving that the strains produced active volatile compounds, they were characterized through a gas chromatography-mass spectrometry (GC-MS) approach, using a novel method that reproduced the dual-plate assay directly in the chromatography vial, which identified a total of 26 different molecules being produced by the growth medium, pathogenic fungus (BC), and the seven strains, grown with or without the fungus (**Table 5, 6, and 7**). Reproducing the conditions is a very important step since many variables can influence the production of volatile compounds and this method allowed us to understand exactly which molecules were produced in the conditions of dual-plate assay. An important thing to note is that, since different production of VOCs has been demonstrated with variation of nutrients available to the microorganisms (Biom *et al.*, 2011; Lazazzara *et al*., 2017), it is entirely possible that the volatile bouquet of these strains is completely different when they are in the environment or inside a plant host, having access to different substrates and with different stimuli, meaning that the *in vivo* and *in planta* contribute of volatile molecules has yet to be accurately assayed.

In general, the pathogen produced very few volatile compounds, not showing any significant difference from the sterile medium. The lack of signature compounds produced by BC made it easier to compare the results obtained from samples containing only the bacterial strain, or the

strain together with BC. This comparison showed that there was never a significant difference between the two (**Table 5**), demonstrating that the strains produce these volatile compounds regardless of a stimulus coming from the pathogen, at least in our experimental conditions.

Also among the bacterial strains there were strains that did not show any unique, signature molecules that differentiate them from other strains (S4C11, R1, and R8) (**Table 6**). In particular, strain S4C11 was very similar to the sterile medium in terms of quantity of the molecules, showing consumption of benzaldehyde and some production of 1-butanol as only differences from the medium, making it not clearly distinguishable as different from the non-treated control. While 1-butanol is reported in literature as an antifungal compound (Batista *et al.*, 2011) it is unlikely that this compound alone is responsible for the inhibition of BC growth, since the levels of production of this strain are comparable to those detected in BC samples. This result asks for further investigation, since the strain managed to inhibit BC growth through volatile compounds. This situation is similar to that of strain R1 and R8, even if the latter is clearly separated from the sterile medium in the PCA analysis (**Figure 11**), resulting overall more distinguishable.

Strains 255-7, 255-8, 260-02, and R16 are instead quite different from the sterile medium, with production of various molecules such as 2-heptanone, dimethyl disulfide, 2-nonanone, cyclodecane, and farnesol.

The ketone progression of 2-pentanone (260-02), 2-heptanone (255-8, 260-02), 2-nonanone (260-02), and 2-undecanone (260-02) has been shown to have an effect on some functions of bacterial cells, interfering with protein folding in *Escherichia coli* cells (Melkina *et al.*, 2017), and with biofilm formation in *Agrobacterium tumefaciens*, expressing also a lethal toxic effect against the latter (Plyuta *et al.*, 2016). Even if effect of these compounds against fungi has not been investigated, it is possible that these compounds might be important in the interaction also between bacteria and fungi.

Also methyl isobutyl ketone (260-02), 1,4-octadiene (260-02), and cyclododecane (255-7) have been reported to have an antimicrobial effect, and this function was detected only in studies on the properties of essential oil of plants (Joo *et al.*, 2010; Han and Bhat, 2014; Lawal *et al.*, 2016; Ganiyat, 2016), making them novel putative biocontrol molecules produced from bacteria.

The key molecules produced by strain R16 are both terpenes: DMNT which is known to be involved in plant defense against herbivorous insects (Tholl *et al.*, 2011), and farnesol which is a known, strong antifungal compound (Shirtliff *et al.*, 2009; Krom *et al.*, 2016).

Apart from the direct antifungal activity, VOCs produced by bacteria are known to enhance plant growth and induce resistance (Tholl *et al.*, 2011; Sharifi and Ryu, 2016), and it is possible that the molecules produced by these strains could have that same effect. This hypothesis can also be supported by the isolation material from which these strains were obtained, consisting of healthy or recovered plants, although the actual volatiles produced *in planta* should be studied to confirm this.

The assays carried out with cultural filtrates also demonstrated the presence of secreted molecules having effect against fungal growth and germination of conidia for BC (**Figure 12 and 14**). Having collected the filtrate after three days of incubation, when the bacteria were in lag phase, the concentration of secondary metabolites and secreted molecules is conceivably at its highest, helped in determining the production of these molecules and their preliminary characterization. Still, further studies can be carried out to determine if a shorter incubation time or a dilution higher than 50% can still have an inhibitory effect. The diffusible molecules include thermosensitive compounds and proteins that, at least in the case of strain S4C11, possibly include lytic enzymes (**Figure 15**). A finer characterization of these molecules will be carried out in future studies, as it is needed to understand the mechanisms underlying this form of inhibition and the chemical nature and stability of these secreted molecules. This last factor could be important for a possible application of these molecules as biopesticides, as the application of molecules can offer several advantages over the whole bacterial cells, such as more standardized efficacy and reducing the unpredictable behavior that living organisms can have under different conditions.

Biocontrol against BC was carried out also on detached plant organ in *in vivo* conditions, demonstrating the ability of the strains to express their activity also in artificial post-harvest conditions which greatly favored the pathogen and the development of the disease (**Figure 16 to 19**). Despite the promising results of some strains, in particular 255-7, in reducing the severity of the disease on tomato berries, actual applicability of these strains in post-harvest conditions must be thoroughly evaluated. For example, strain 255-7 belongs to the *Pantoea agglomerans* species, which includes some strains which are pathogenic for humans or plants (Cruz *et al.*, 2007).

Therefore, before use in realistic conditions, all doubts concerning the safety of the strain need to be addressed. Other important practical points that should be addressed to understand if these strains can be actually useful as a base for a biocontrol product can be assessing their ability to control a wide range of pathogens, determining their genetic stability, and production features such as being suitable for low-input production and formulation to increase shelf-life. Also, should they prove to be sufficient for biocontrol in these conditions, volatiles produced by the strains could be employed to avoid most these concerns, obtaining the desired effect without exposing the crop to the microbial cells themselves and their possibly unforeseen behavior.

The results of the *in vivo* assays demonstrated the ability of the strains to act against a phytopathogenic fungus in association with a natural substrate, not just in artificial medium, and encouraged the *in planta* phase of the assays.

For some of these strains (255-7, 260-02, and R8), it was possible to label them with a plasmid carrying a fluorescent protein and a resistance to kanamycin, allowing to understand their interaction with several plant hosts. Despite having been isolated from woody plants, these strains demonstrated an ability to colonize endophytically herbaceous plants of different families, having been successfully recovered from the leaf tissues of these plants after having been inoculated at the root.

Among these, a particular association was studied more in-depth through the use of confocal microscopy: the interaction between strain 260-02:*gfp* and pepper (*Capsicum annuum* var. Zebo F1), and compared with the interaction between a pathogenic *P. syringae* strain DC3000::*gfp* and these plants.

It was possible to see a different behavior of the two strains in their interaction with the plant root, with strain 260-02 favoring the surface of primary roots and strain DC3000 favoring secondary roots, possibly damaged ones, and proceeding with excessive colonization when possible (**Figure 21**).

Also, it is of note that these experiments were not carried out on sterile soil, but on normal potting soil. During the time that passed from the sowing of the seeds to the seedling stage, the plants had time to establish a rhizosphere community, since previous studies examined rhizosphere community as early as seedling stage (Chaparro *et al.*, 2014). Strain 260-02:*gfp* managed to

integrate itself with this community, while strain DC3000::*gfp* being found prevalently in damaged, weaker, and newer tissues seems to indicate that it is not capable of integrating in a functional rhizosphere community.

While the strains were able to colonize the plant hosts, not all the interactions were positive, with some being neutral despite colonization (**Figure 22, 24, 27**). This suggests that the plant-growth promoting effects of these strains do require a particular interaction between the bacterial cells and the plant host to express the positive effect.

Based on these tests on multiple hosts, as well as the pathogenicity tests for the selected pathogens, pepper was chosen as the plant material to use for all the *in planta* assays.

The different strains had different effects on the growth of pepper, even if all of them, with the exception of strain R16, managed to promote growth compared to the non-treated control. For the strains 255-7, 255-8, 260-02, R1, and R8 promotion of growth is assessed at the end of the assay as an increase of plant height of approximately 20% (**Figure 29**), which is in line with previous results obtained with *C. annuum* plants in greenhouse conditions (Kang *et al.*, 2006; Zhang *et al.*, 2016), while strain S4C11 caused an increase of almost 40% in plant height, exceeding these previous results.

This result partially correlates with the biochemical analyses for traits typical of PGPR that were previously carried out on the bacterial strains: strain R16 showed no growth promotion ability and lacks all the assayed PGPR traits except for production of auxins (siderophore production, phosphate solubilization, ACC-deaminase), which is still produced at lower levels than in other strains (data not shown). Still, there is no clear indication from these biochemical traits to identify a main trait of importance as the other strains have a varied pattern regarding these traits and manage to promote growth all the same. In particular, strain S4C11 shows only production of auxins (at a higher level than R16 but not the highest) and production of siderophores among these traits, and has the best growth promotion effect.

It is also important to note that the time when the bacteria are applied to the plants can affect the results of plant-growth-promotion. Preliminary studies carried out on strain R16 confirmed that it does not promote growth of pepper plants when inoculated 2 weeks after germination, but showed a significant increase in height when inoculated a month after germination (data not shown),

suggesting that different methods and timing of inoculation could further increase the results obtained even by other strains.

Also, the strains induced precocious flowering by 7-10 days (data not shown), a phenomenon that was previously reported for plant-PGPR interactions with plants, such as *Arabidopsis thaliana* and *Panicum virgatum* in association with *Burkholderia phytofirmans* strain PsJN (Poupin *et al.*, 2013; Wang *et al.*, 2015).

These preliminary results on plant-growth promotion are promising, but require some additional investigation. In particular, the effect on actual yield of the plants should be estimated in a longer assay that allows the plants to produce fruits.

The results obtained in the plant-growth promotion assays indicated a positive interaction between most of the strains and the plant hosts but do not disclose the strains' ability to have an effect on plant health. For this reason biocontrol assays were carried out to determine if the strains could reduce the severity of diseases caused by a fungal pathogen (*R. solani* strain RS1), a bacterial pathogen (*P. syringae* strain DC3000), and a viral pathogen (*Cymbidium ringspot virus*).

The fungal pathogen RS gave inconsistent results throughout the different trials as it always reduced the height of infected plants, but could increase the weight, rather than reduce it (**Figure 31**). In these cases, the plants also had a very dark green coloration and did not look unhealthy, apart from displaying stunted growth. Regardless of the phenotype induced by the pathogen, the bacterial inoculation did not seem to have any relevant effect in contrasting the pathogen. There is also the possibility that the standardization of the protocol used for the different pathogens, that included root dipping at two weeks after germination, is unsuitable for the pathogen of seedling damping-off, leaving the plants too mature to develop the typical symptoms of the disease.

Biocontrol against *P. syringae* DC3000 was detected for all the examined strains, but with different efficacy: some strains (255-7, 260-02) could reduce the number of necrotic spots developed on pepper leaves seven days after the inoculation of the pathogen; some strains (S4C11) managed to reduce the number of necrotic spots measured 14 days after the inoculation of the pathogen; the remaining strains (255-8, R1, R8, R16) reduced the number at both measurements. These different patterns suggest that the mechanisms underlying this effect are not the same for all strains. In particular, the effect of strain 260-02 was investigated more in depth. Through confocal

microscopy, it was possible to see that plants that were inoculated at the root with strain 260-02 showed less colonization by strain DC3000::*gfp* on the leaves compared to a non-treated control inoculated with the same pathogen (**Figure 33**). Since the inoculation of the pathogen was carried out one week after the root dipping of the plants in this experiment, it is unlikely that the bacteria managed to reach the leaf tissue to interact directly with the pathogen, and it is therefore more plausible that strain 260-02 had an indirect effect, activating the defenses of the plant against bacterial pathogens. Induction of plant defenses by treatment with beneficial bacteria is a mechanism that has been reported for several strains of Bacillus and Pseudomonas and can be employed also by other bacteria (Durairaj *et al.,* 2018). It is also interesting to notice that, while DC3000 is a leaf pathogen, it had a detrimental effect on plant defenses when inoculated at the root, allowing for a much higher rate of colonization by DC3000::*gfp* on the leaves (**Figure 33**).

A viral pathogen was employed during the biocontrol assays to analyze how the bacterial strains would interact with a pathogen against which direct biocontrol mechanisms cannot be effective, allowing to investigate indirect mechanisms that might be at work. Out of the 7 bacterial strains, only 3 managed to significantly reduce the symptom severity of pepper plants inoculated with *Cymbidium Ringspot Virus* (CymRSV). It is interesting to note that these strains (255-7, 260-02 and R16) showed different results in the other assays, showing a different antifungal effect both *in vitro* and *in vivo* (**Figures 8, 9, 10, 12, 13A, 16, 19**), producing much different volatile molecules (**Figure 11**) and affecting differently the growth of pepper plants (**Figure 29**). Also the analysis of relative gene expression discloses little similarities between the effect that these strains have on the host plant (**Figure 36**), strengthening the hypothesis that, despite the similar result achieved, the mechanisms underlying the biocontrol of the viral pathogen is not the same.

It is interesting to note that in the plants treated with strain 255-7 the concentration of the virus is much higher than in other treatments, despite the low symptom severity registered. If this result was to be confirmed even for other viruses or hosts, this strain could be unsuitable for actual biocontrol against viral pathogens since infected plants, even if asymptomatic, can still act as source of inoculum for these pathogens. A similar result, obtaining plants with lower or no symptoms but not lower pathogen concentration was already reported for daisy plants infected with chrysanthemum yellows-associated phytoplasma and treated with *Pseudomonas putida* (Gamalero *et al.*, 2010)

The substantial reduction of symptoms observed in the plants treated with strain 255-7 could correlate with the decrease in methylation levels registered in plants treated with this strain (**Figure 30**). As methylation of cytosine in eukaryotic DNA is part of gene silencing, the reduction of this methylation suggests a general higher level of gene expression, at least at a translational level (Berger, 2007). Also, if the plants have a generally more active translational mechanism thanks to the treatment, it is conceivable that also the virus replicates faster, possibly explaining the higher concentration registered in those plants.

During the different experiments carried out in this study, the plant-growth promotion and biocontrol traits of the examined strains became evident and brought us to inquire on the genetic basis that could underlie these traits. This question was especially interesting for strain 260-02 which, according to sequencing of the 16S rRNA gene, belongs to the species *Pseudomonas syringae*, known for being one of the most widespread pathogens worldwide.

Since this species has been extensively investigated throughout the years, also at a genomic level, there were plenty of resources to use to compare the genomes of different pathogenic strains with that of our beneficial one through a pangenome analysis aimed at characterizing the difference in gene content between pathogens and strain 260-02.

This analysis identified very few different genes, particularly in comparison to strain UMAF0158, the closest relative to strain 260-02 which was characterized as a pathogen from mango plants (Arrebola *et al.*, 2007).

The identified different genes could be gathered in a few categories of putative relevance to pathogenicity: toxins/effectors, quorum sensing, type IV secretion system, and gene expression (**Table 14**). The relevance of these genes must still be investigated, but the gene expression category seems a promising one, as a previous study showed how the gene content differences between closely related beneficial and pathogenic species were minimum and not in key genes related to interaction with the host, opening perspectives in how gene expression might be different instead (Sheibani-Tezerji, 2015). This hypothesis is also reinforced by the fact that, in many cases, the same genes and traits that are essential to beneficial endophytes, such as those related to colonization of the host, production of hormones, and utilization of resources from the host, are the same as those present in pathogens (Gourion *et al.*, 2015; Kroll *et al.*, 2017).

A great limit of these pangenome analyses is the presence of many genes which function is not known in the genomes. Even for a well-characterized species such as *P. syringae*, there are thousands of genes that have no known function assigned to them, and these genes cannot be properly assessed with this kind of analysis. There were several of these genes, which product is characterized only as hypothetical protein, that had different presence among pathogenic strains and strain 260-02, still there is no way to understand the function of these genes and their effect on pathogenicity, if any, from a mere genomic analysis. For the other strains, a draft sequence of the genome was obtained as well, and some genes related to the traits observed during *in vitro* assays could be identified. Still, the amount of data that could be obtained from the *in silico* analyses was little, in some cases because of the lack of resources on some relatively unknown species or genera, such as for *Paenibacillus pasedenensis* or *Lysinibacillus fusiformis*.

In conclusion, this study sought to investigate the effect of 7 putative endophytic strains in biocontrol and plant-growth promotion assays, as well as highlight the interactions underlying these effects, identifying three main levels of interaction that were identified but require further study to fully characterize: (i) molecular cross-talk, (ii) colonization of the host, and (iii) alteration of host's gene expression.

Molecular cross-talk between the biocontrol strain, the pathogen, and the host is surely involved in the direct biocontrol observed during *in vitro* and *in vivo* assays and possibly involved also in the effect on the host plants. The production of volatile compounds, some of which already reported for these effects, is a clear indication of some sort of molecular signaling exerted by the bacteria. Some points still remain to be explored, such as the role of non-diffusible molecules. Also, it is interesting to note that it seems to be no different production of volatile molecules when the strains are in the presence of the antagonized fungus. This point will be addressed in further studies, determining if the physical proximity of the pathogen can be a stimulus for the production of different molecules. Likewise, it is still unknown if the bacteria can change the molecules they produce in response to stimuli coming from the plant host.

A second level of interaction (ii) can be identified in the colonization of the host. The seven strains used in the study were isolated as putative endophytes and, having recovered from the leaves cells of the strains after inoculation of the root, the endophytic lifestyle of these bacterial strains seems to be confirmed, even if the final proof of the endophytic nature of these strains can

only come from detection inside distal plant tissue by microscopy. The successful colonization ability of these bacteria was shown by inoculating plants that were grown for 2 weeks in a non-sterile environment: during this time, the seedlings already developed a rhizosphere community and, to have an effect on the plants, the analyzed strains must have managed to successfully establish themselves in this community. An interesting aspect to investigate, related to colonization, is the effect that the strains have on the microbiota of the host plant, analyzing in depth what changes they bring in the microbial community established in the plants, and if this change can be correlated to the effect that is registered.

The third level of interaction (iii) was identified in the modification of gene expression in the host. This phenomenon has been extensively studied both in beneficial bacteria, in particular in relation to induced systemic resistance (Iavicoli *et al.*, 2003; Verhagen *et al.*, 2004), and in pathogens (Jamir *et al.*, 2004; Thilmony *et al.*, 2006), showing that microorganisms that evolved to live in association to plants are capable of modulating the expression of genes of their hosts to better suit their needs. In this study, some different gene expression was registered for plants treated with the bacterial strains and then inoculated with a viral pathogen. Furthermore, a significant difference in methylation level of cytosine was detected in the genome of plants treated with one of these strains. These results open further perspective for the study of the effect that these strains have on the host, using -omics approaches to see the full picture of what is going on in these plants, at a transcription and translation level, and possibly also identifying the molecules – proteins or RNAs – that are directly causing this phenomenon.

This study, evaluated the same strains through *in silico*, *in vitro*, *in vivo*, and *in planta* assays, and obtained different and at times contradictory results throughout the different levels of experiments. The *in vitro* and *in silico* analyses, having the most controlled conditions, yielded results that did not always correlate to those seen in greenhouse, which may yet be different from those that will be obtained in assays carried out in full field conditions. These results confirm the crucial importance of interaction between plant, pathogens, environment, and beneficial bacteria towards achieving effective biocontrol and identify interesting aspects to investigate in future studies.

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