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"Biocontrol strategies against plant pathogens"

By

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Declaration

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly with due reference to the literature and acknowledgement of collaborative research and discussions.

This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

Signature ***Date*** 14-06-2021

Dedication

This thesis is wholeheartedly dedicated to my parents, ***Amatul Hafeez*** *and* ***M. Rafiq Shahzad****, especially to my endearing mother, who have always been my source of inspiration and gave me strength of staying on my feet and not giving up on achieving high goals of life.*

To my ***siblings,*** *who shared their words of advice and encouragement to finish this study.*

And lastly, to my late friend, ***Taimoor Akhtar****, who always encouraged me to accomplish the ambition I have been nourishing since long.*

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Gul-i-Rayna Shahzad

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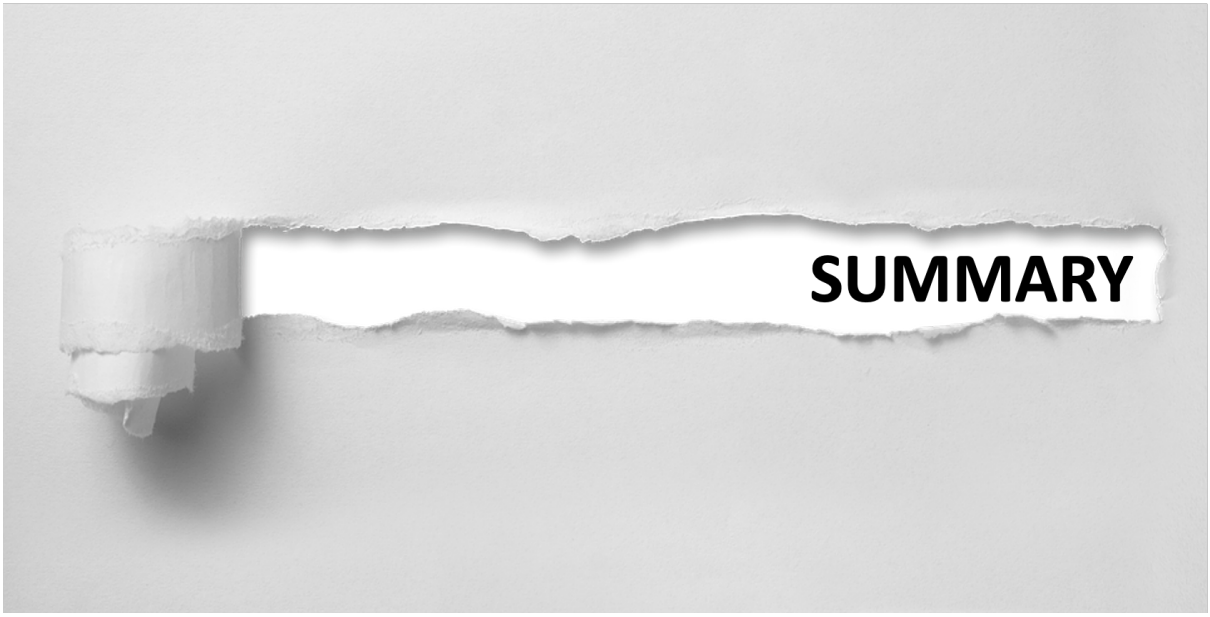
SUMMARY

Summary

It is a known fact that the whole agriculture system is suffering from the diseases caused by plant pathogens, affecting negatively the crop yield production, food security, biodiversity, agricultural ecosystem and hence agricultural economy. In many countries, the containment strategies of plant pathogens are still depending on chemical pesticides that cause adverse effects in the long term. According to the implementations reinforced by European council 2009/128/EC, biocontrol strategies are considered as the most profound and integrated approach for sustainable disease management. Defining biocontrol in terms of plant pathology, it is the purposeful utilization of beneficial microbes, or its molecules, to suppress phytopathogens' ability to colonize or induce symptoms in the host. In spite of their lesser shelf-life and unreliability as compared to conventional pesticides, their targeted biological interaction with the phytopathogens reduces the possibility of affecting non-target organisms, environment and the development of resistance in the pathogen.

In this context, exploitation of bacterial endophytes has gained much attention during the past decades. Endophytic plant growth promoting bacteria (ePGPBs) mediate their biocontrol efficacy by targeting species through a multitude of direct or indirect biological interactions, often employing both modes of action, such as plant growth promotion, host's resistance induction, allelochemicals secretion, and nutrients and niche competition. Another strategy that has gained popularity is the exogenous application of double stranded RNA (dsRNA), which is considered as the key trigger molecule of RNA interreference (RNAi), a post-transcriptional gene silencing mechanism, and has been shown to provide protection without the need for integration of dsRNA-expressing constructs as transgenes.

In the present doctoral thesis, the above-mentioned biocontrol strategies were adapted, utilizing "ePGPBs as microbial inoculants" and "exogenously applied dsRNA as RNAi based natural product", against several phytopathogens belonging to different families of viruses and fungi.

Regarding ePGBPs as microbial inoculants, the objective of this study was to extend our understanding of five endophytic bacterial strains; *Pantoea agglomerans* (255-7), *Pseudomonas syringae* (260-02), *Lysinibacillus fusiformis* (S4C11), *Paraburkholderia fungorum* (R8), *Paenibacillus pasadenensis* (R16); that have shown a promising result in previous studies. In the present doctoral study, these strains were tested *in planta* to evaluate their role in providing plant growth promotion and broad-spectrum protection against two target pathosystems (viruses and fungi) that might have direct, indirect or simultaneous effects,

proceeded with two following aims: (Aim 1) action against viruses: *Cymbidium ringspot virus* (CymRSV), *Cucumber mosaic virus* (CMV), *Potato virus X* (PVX), and *Potato virus Y* (PVY) on *Nicotiana benthamiana* plants, comparing their effects with those of three chitosan-based products, which are known to induce resistance in plants; and (Aim 2) action against fungal pathogens: *Rhizoctonia solani*, *Pythium ultimum and Botrytis cinerea* on *Lactuca sativa* plants, comparing their effects with *Bacillus amyloliquefaciens* strain (CC2) and *Trichoderma* spp. based product, under controlled conditions.

To test the priming efficacy of ePGPBs against target viruses, several phenotypic parameters were observed along with the evaluation of three plant defense related genes (EDS1, PR2B and NPR1) on *Nicotiana benthamiana* plants. Interestingly, the symptoms reduction was successfully registered against CymRSV and CMV with increased heights of the plants. Some of the treatments were shown correlation between severity of symptoms and the virus concentration in the plants. Furthermore, the molecular interaction indicated the involvement of a salicylic acid (SA) mediated defense pathway as evidenced by the increased expression levels of EDS1 gene in strains R16 and 260-02. Whereas, strain S4C11 showed downregulation of PR2B gene, suggesting that SA-independent pathways could be involved. These findings opened queries regarding the duration of the protective effect, host-plant-pathogen interaction, and epidemiological implications of the use of similar biocontrol strains, that reduce the symptoms but not the concentration of virus in the host.

To test the ePGPBs role against target fungi (pre- and post-harvest stage), several experiments were conducted including phenotypic paraments, gene expression analysis (PR1, PAL, ThlP3, ERF1 and ACCS1), microbiota analysis in bulk soil, rhizosphere, and root associated with *Lactuca sativa* in the presence or absence of the inoculants, and nutritional quality parameters at time of harvest and during shelf-life of Romaine lettuce. The results were accompanied in terms of symptoms reduction by strain R16 (*P. ultimum*, *R. solani*, *B. cinerea*) and strain 260-02 (*R. solani*, *B. cinerea*); % seed germination by strains R16, 260-02, 255-7, S4C11 in some healthy and *R. solani* infected lettuce varieties; inhibition of *R. solani* population in soil and rhizosphere soil by strains R16, 260-02 and 255-7. Furthermore, composition of the bacterial microbiota was radically different in the rhizosphere and the root endosphere among treatments, while the bulk soil formed a single cluster regardless of treatment, indicating that the use of these treatments did not have an ecological impact outside of the plant. Also, these strains were able to contribute to the maintenance of nutritional quality indexes of lettuce at harvest and during storage. All the obtained results indicated that these

strains were involved directly (via antibiosis) and indirectly (via SA or ET/JA) in the observed reduction of symptoms. Particularly, strain R16 upregulated both PAL and ACCS1 gene in *R. solani* infected *L. sativa* (suggesting co-activation of SA- and JA/ ET mediated ISR resistance); strain 260-02 upregulated PAL gene in *R. solani* infected romaine lettuce and showed higher levels of ascorbic acid (AsA) production in *B. cinerea* infected romaine lettuce (suggesting the activation of SA- and AsA-mediated antioxidant resistance); and strain 255-7 triggered PAL and ThlP3 gene up-stream expression levels indicating SA mediated pathways in *R. solani* infected romaine lettuce. These findings affirmed the previous conclusions and added valuable pieces of information regarding the traits these ePGPB carried, most importantly, in individuating different mode of action of the different strains in different host plants with or without the presence of pathogen.

Regarding the second approach, non-transgenic strategy was employed to induce resistance against *Tomato Aspermy Virus* (TAV) in *N. benthamiana* plants. DsRNA molecules for coat protein (CP) gene was produced by a two-step PCR assay followed by *in vitro* transcription and purification and was exogenously applied. The implementation of CP-derived dsRNA TAV was not successful in reducing observed symptoms (mosaics, blisters, crinkling, leaf distortion, and systemic vein clearing), regardless of treatments or days of post inoculation. Only a slight difference was found in plant heights indicating that the treatment managed to reduce stunted growth of the plant at dilution 01:10 (6 and 12 dpi). The reasons could involve inappropriate concentration of dsRNA inoculum. Therefore, future studies will be conducted to optimize *in vitro* dsRNA molecules production to obtain higher concentrations or more specific sequences, and more suitable viral genes.

Both strategies have shown interesting outcomes and gave us the future direction which will help us in designing the adequate trials (*in planta* or semi-field) for the disease management and diseases control through the application of ePGPBs as a microbial inoculants and dsRNA-based product individually or in combination.


GENERAL INTRODUCTION

General Introduction

Throughout their lives, plants are consistently challenged by a range of unfavorable environmental factors (Walters, 2010). Unlike other living organisms, plants' sedentary lifestyle does not allow them to get away from these adverse factors and therefore made them utilize various defense strategies for their survival. They possess multifunctional defense strategies that not only allow them to resist against the attack of pathogens but also enable them to respond against abiotic stresses (Zaynab et al., 2018). Biotic agents that cause vulnerability in plants include pathogenic microorganisms, such as fungi, bacteria, protozoa, viruses and nematodes, whereas abiotic stresses include toxic chemicals present in soil, water or air, lack of micro/ macro-nutrients, light and moisture.

Upon sensing biotic stress, plants activate potential coping mechanisms generally termed as plant innate immunity via hormone signaling pathways that are regulated by Jasmonic acid (JA) or Ethylene (Et) and Salicylic acid (SA) mediators, either alone or in combinations, acting as two strategic ways. The first uses the fundamental form of protection through the pattern recognition receptors (PRRs), located on the external surface of the plant cell to recognize conserved pathogen (or microbe)-associated molecular patterns (PAMPs or MAMPs) like fungal chitin, flagellin, bacterial lipopolysaccharides, peptidoglycans and quorum sensing molecules. These receptors are mostly leucine-rich repeat kinases and lysine motif (LysM) kinases and have role in intracellular signaling, transcriptional reprogramming and triggering of PAMP-triggered immunity (PTI) (Monaghan and Zipfel, 2012; Zhang et al., 2018). Certain successful pathogens secrete their PTI-inhibiting virulence factors, typically known as effector proteins. to elude PTI, facilitate nutrient acquisition and promote pathogen dispersal by causing disease that is termed as effector-triggered susceptibility (ETS) (Pieterse et al., 2009). The plant surveillance system on the other hand have evolved a second class of receptors, resistance (R) proteins, which detect particular effectors proteins modulated by pests and pathogens and stimulate the plant defense responses via a mechanism known as effector-triggered immunity (ETI). The receptors of R proteins encode nucleotide-binding leucine-rich repeat (NLR) intracellularly and target specific effector proteins either directly as a receptor and ligand or as modified decoy of target to induce ETI following the regulation of antagonistic defense hormones (Fig. 1) (Backer et al., 2019; Spoel and Dong, 2012).

While plants have many natural defenses to withstand and react to pathogen attack, the entire physiology of the plant becomes disoriented and leads to consequent destruction once the plants lower their guard (Dvořák, 2009).

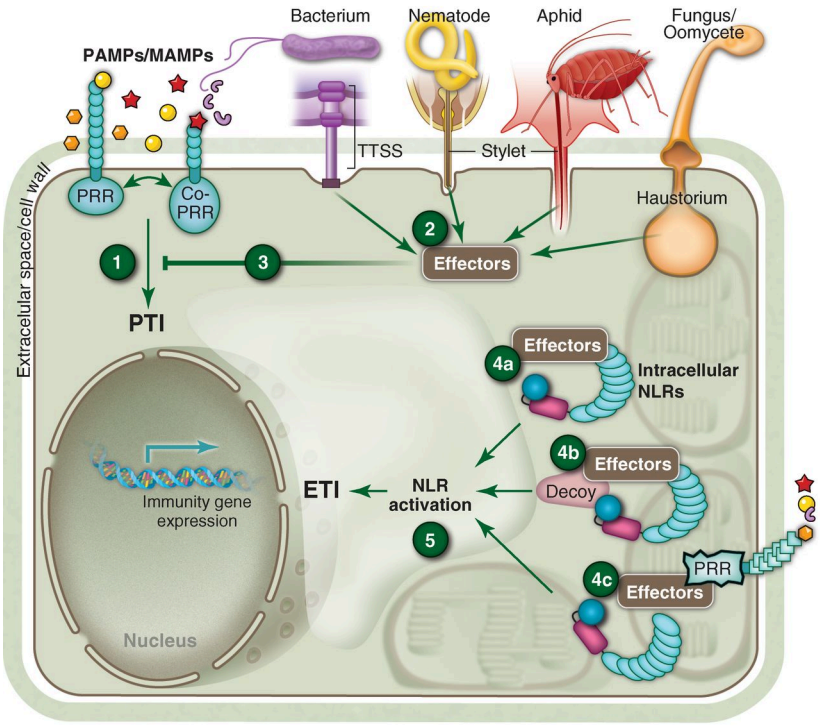

Figure 1. Schematic illustration of plant immune system (Dangl et al., 2013)***.*** *Steps: (1) Plants perceive these via extracellular PRRs and initiate PRR-mediated immunity (PTI); (2) Pathogens deliver virulence effectors to both the plant cell apoplast to block PAMP/MAMP perception (not shown) and to the plant cell interior; (3) These effectors are addressed to specific subcellular locations where they can suppress PTI and facilitate virulence; (4a) Intracellular NLR receptors can sense effectors in three principal ways: first, by direct receptor ligand interaction; (4b) by sensing effector-mediated alteration in a decoy protein that structurally mimics an effector target, but has no other function in the plant cell; and (4c) by sensing effector-mediated alteration of a host virulence target, like the cytosolic domain of a PRR.*

Over the years, agriculture has been the basis for the survival and growth of mankind however, in the 21st century, the concerns regarding modern agriculture became progressively more dire because of the expanding population and limited resources to produce food in a profitable, sustainable, and efficient way. The production of agricultural crop plants is at stake mainly due to the limitations caused by biotic factors which is the most challenging concern to deal with, that accounts for almost 31-42% yield loss worldwide (Moustafa-Farag et al., 2020). For instance, these limitations can involve the sudden introduction of an alien pathogens against which the plant has no previous interaction and cannot trigger effective defenses; pathogens invade the plants by following some attractive traits present in plant species; and the selection of genetic traits carried out during domestication that brought wild plants to become the crops we know nowadays, which tend to focus more on the quantity and quality of the produce the crop could yield and, as a result, often ignored defense traits. By considering these facts, it becomes evident that plant diseases are a main threat for agro-ecosystem, food security and safety. Even the slightest negligence can cause a drastic reduction in the production of crops.

The area of focus of the present doctoral thesis was centered on phytopathogens, particularly, viruses infecting Solanaceae family and fungal (pre-/ post-harvest pathogen) infecting Asteraceae family. Their details are discussed in below-mentioned sections 1.1 and 1.2.

1.1. Plant viruses attacking nightshade family, *Solanaceae*

The nightshade family (Solanaceae) is a monophyletic dicot group of cultivated crops that has shown an essential role in human nutrition and traditional medicine (Afroz et al., 2020). After Poaceae and Fabaceae, it is considered as the third most important family of angiosperms that consists of about 2700 species and 98 different genera (Olmstead and Bohs, 2006). It contains approximately 1200 species of the most important cash crops belonging particularly to the genus *Solanum* L. which is widely distributed all around the world (Knapp et al., 2019). Such as, *S. melongena* L. *subsp. melongena* (eggplant), *S. lycopersicum* L. (tomato), *S. tuberosum* L. (potato), and *S. muricatum* Aiton (pepino) (Ghatak et al., 2017). Besides crops, *Atropa belladonna* (deadly nightshade), *Datura stramonium* (Jimson weed), *Hyoscymus niger* (black henbane), *Nicotiana* sp. (tobacco) and *Capsicum annuum* (pepper) are among the well-known medicinal plants and have been consumed as herbal medicine over the centuries (Chowański et al., 2016). Furthermore, the key bioactive secondary metabolites found in the Solanaceae family include alkaloids, flavonoids, glycosides, lactones, lignans, anti-microbial peptides, sugars, steroids, terpenoids and simple phenols (Ghatak et al., 2017). These compounds have had several implementations in different traditional medical systems like Ayurveda, Siddha, homeopathy, Unani and Traditional Chinese Medicine (Chowański et al., 2016), and were also utilized as antimicrobial, insecticidal, anti-infectious agents and as poisons (Shah et al., 2013; Tamokou et al., 2017).

However, in all dynamic cultivation contexts, Solanaceous crop plants are vulnerable to pathogenic attack, including viruses.

Plant viral infections lead to substantial losses in yield and fruit quality, adversely affecting human well-being due to agricultural and economic losses, which may have implications also for biodiversity conservation (Souiri et al., 2020). Historically, positive single-stranded RNA tobacco mosaic virus (TMV) was the first virus discovered in tobacco, a *Solanaceae* (Beijerinck, 1942). There are currently 1901 recognized plant virus species being categorized into 6 orders, 32 families and 141 genera (ICTV Master Species List 2018b.v2., 2019). The entire genome of plant viruses is protected by coat proteins where they carry single or double strand RNA/DNA. In addition, the genome size and shape varies based on their virus particles such as isometric or rod-shaped, gemini, etc. (Abdelkhalek and Hafez, 2020). Their mode of transmission is either horizontal or vertical, the former being facilitated by vectors or by contact, the latter by means of seeds or pollens (Hančinský et al., 2020). In most cases, viruses infecting Solanaceous crop plants are transmitted by insect vectors, particularly aphids transmitting potato leaf roll virus in potato varieties (Mondal et al., 2017), thrips with tospoviruses (Rotenberg et al., 2015), whiteflies with torradoviruses (Verbeek et al., 2014), nematodes transmitting tobacco rattle virus (Riga et al., 2009) and so on. In contrasts, there are few examples of vertical transmission in viruses infecting Solanaceae species: most notably tobamoviruses (Dombrovsky and Smith, 2017), pepino mosaic virus (Hanssen et al., 2010), and tomato torrado virus (Pospieszny et al., 2019). Once they enters inside the plant, they replicate in individual cells by modifying and using host's replication machinery to spread progressively and systemically between the cells and vascular systems (Abdelkhalek and Hafez, 2020).

The present work focused on the following viruses that can infect Solanaceae plants: *Cymbidium ringspot virus*, *Cucumber mosaic virus*, *Potato virus X*, *Potato virus Y*, and *Tomato aspermy virus*.

Cymbidium ringspot virus (Tombusviridae)

Cymbidium ringspot virus (CymRSV, genus *Tombusvirus*) was first discovered by Hollings and Stone on *Cymbidium* spp. in early 1960s. Its virions are found in leaves, stems, flowers and roots, and comprises of non-enveloped and isometric particles with a diameter of 30nm. The virus genome is a monopartite, positive single stranded RNA 4.733kb long. It naturally infects host species of *Cymbidium* spp. and *Trifolium repens,* however, its mode of transmission in host species often occur due to mechanical means or by the contact between plants. The occurrence of CymRSV is distributed all over the UK including some countries of Europe where it systemically infects the host families of Solanaceae followed by Leguminosae-Papilionoideae*,* Compositae*,* Chenopodeaceae*,* Cucurbitaceae and Amaranthaceae (Hollings et al., 1977). The common symptoms of this virus include chlorotic lesions, mottling, necrotic ring spots, leaf curling and stunted growth of susceptible plants.

Cucumber mosaic virus (Bromoviridae)

Cucumber mosaic virus (CMV, a type member of genus *Cucumovirus*), is an icosahedral, positive single-stranded RNA virus with a diameter of approximately 28-30nm. It was first reported on *Cucumis sativus* and other members of Cucurbitaceae family (Doolittle, 1916; Jagger, 1916). The total size of CMV genome is 8.621kb which is designated to three genomic parts i.e. RNA1, RNA2 and RNA3 (Jacquemond, 2012). RNA1 encodes the 1a protein having methyltransferase and helicase motifs that is requisite for viral replication. RNA2 encodes two proteins, in which, 2a protein contains RNA-dependent RNA polymerase (RdRp) domain, whereas, the 2b protein comprehends RNA silencing suppressor activity. RNA3 encodes proteins 3a and 3b, the coat and movement proteins that are required for the movement and transmission of virus (Jacquemond, 2012). CMV isolates have worldwide distribution including both temperate and tropical regions, having been reported in over 1200 species belonging to more than 100 families of monocots and dicots, including vegetables, fruit crops, weeds and ornamentals (Miozzi et al., 2020). CMV can be transmitted via three means in a non-persistent manner; first is the vector transmission that occurs by more than 75 species of aphids, second transmission occurs by mechanical means and the third transmission occurs through the seeds (Palukaitis and García-Arenal, 2003). CMV has been ranked as number 4 among the top ten economically important viruses, causing diseases more frequently in the major crop families of Solanaceae, Leguminosae-Papilionoideae, Cruciferae, Compositae, Chenopodeaceae, Cucurbitaceae and Amaranthaceae, and displaying symptoms such as necrosis, dwarfing, chlorosis and malformation mostly depending upon the host species (Rybicki, 2015).

Potato virus X (Alphaflexiviridae)

Potato virus X (PVX, genus *Potexvirus*) was first described by Smith in (1931) on a solanaceous crop *Solanum tuberosum* (potato) in the UK. PVX is composed of linear, monopartite, single stranded positive sense RNA with a genome size of approximately 6.4kb. The genome has a non-enveloped and filamentous flexible structure of about 515nm in length and 13nm in diameter (Huisman et al., 1988). It comprises five open reading frames (ORFs) encoding five proteins: RNA-dependent RNA polymerase (RdRP), three overlapping ORFs encoding movement proteins that form the Triple Gene Block module (TGBp1, TGBp2, and TGBp3), and the coat protein (Morozov et al., 1991). Furthermore, the virion has highly hydrated surface, deeply grooved and helical symmetry (Parker et al., 2002). PVX is considered among the top ten most economically important RNA virus (King et al., 2011). It has worldwide distribution, predominantly in the potato growing areas, and the disease is spread either by grasshoppers or biting insects, often via mechanical contact, and by agricultural equipment such as tractors or sprayers (Ephytia Inrea, 2020). PVX has a broad natural host range, with at least 62 plant species from 27 family members. Besides major Solanaceae crops

(like tobacco, tomato, potato, bell pepper, and chili), PVX infects the members of Chenopodeaceae, Amaranthaceae, Compositae, Fabaceae, Vitaceae, Brassicaceae and Daisy family (CABI, 2020), and causes symptoms of interveinal chlorosis, mosaic patterns, leaf crinkling, acute tip necrosis and stunted growth.

Potato virus Y (Potyviridae)

Potato virus Y (PVY, a type member of genus *Potyvirus*) was first discovered on a Solanaceous crop *S. tuberosum* causing potato degeneration, a disorder known since the 18th century (Smith, 1931). PVY has a long filamentous particle that carries a monopartite, single stranded, positive sense RNA genome of about 9.7kb (Adams et al., 2005). As in all potyviruses, the genome of PVY contains poly(A) tail at 3' terminus and a covalently linked VPg protein at 5' terminus that have roles in the protection, replication and regulation of genome expression (Karasev and Gray, 2013). The strains of PVY have been distinguished based on pathotypes and phylogroups using biological and gene sequence differences, usually being recognized as PVY^{O} (common strain causing mosaic symptoms in most hosts), PVY^{C} (stipple streak strains), PVY^{N} and PVY^{NTN} (tobacco veinal necrosis strains causing severe necrosis referred to as potato tuber necrotic ringspot disease, PTNRD) (USDA APHIS, 2020). Transmission of PVY strains occur mainly by the insect vectors belonging to at least 70 *Aphididae* species in a non-persistent manner. Second transmission routes include mechanical spread of the virus and the third means of transmission is through seed contamination, which is remarkably high in seed-grown potatoes. Besides being a worldwide pathogen of potatoes, PVY causes economic damages to Solanaceous crops species such as pepper, tomato, eggplants and tobacco (Jones, 2014; Kerlan, 2006). Also, PVY has a wide host range including the plants in Chenopodeaceae, Amaranthaceae families causing symptoms of mild to severe mosaic or mottling, crinkling, and stunted growth (Ephytia Inrea, 2020).

Tomato aspermy virus (Bromoviridae)

Tomato aspermy virus (TAV, genus *Cucumovirus*) was first reported in tomatoes by Blencowe and Caldwel in the year (1949), and was later identified as the cause of flower distortion in *Chrysanthemum morifolium* (Prentice, 1951). The total TAV genome of 8.698 kb consists of linear, single stranded positive RNA (a tripartite, RNAs 1, 2 and 3 encoded with five open reading frames). RNA 1 and RNA 2 encode the virus-replicating 1a and 2a proteins (Palukaitis and García-Arenal, 2003). Whereas, the overlapped 2b protein from sub-genomic RNA4 facilitates long distance movement and influences virulence by suppressing plant-host mediated gene silencing (Li et al., 1999). Furthermore, the dicistronic RNA3 encodes 3a movement protein and capsid protein that is expressed from the structural protein of subgenomic RNA4, responsible for virus mobility in host plants, insect transmission, expression of symptom and host range (Kaplan et al., 1998; Palukaitis et al., 1992). TAV is transmitted in host plants in a non-persistent manner via three modes:

mechanical inoculation, seed contamination and transmission via insect vector operated by Aphids (Gupta and Singh, 1981). TAV infects broad range of plants belonging to dicots and monocots families such as Solanaceae, Leguminosae-Papilionoideae, Chenopodeaceae, Liliaceae, Cucurbitaceae, Cannaceae, Compositae, and so on. The symptoms vary from plant to plant depending on the persistence such as mild to severe mosaics patterns, malformation, seedless fruits, dwarfing, and deformation of flowers (Raj et al., 2007).

1.2. Fungal pathogens attacking daisy family, *Asteraceae*

To date, Asteraceae (Compositae) is known as the largest family of angiosperms, that comprises of 32,913 accepted species belonging to 1911 genera and 13 sub-families (TPL, 2020; WCVP, 2020). Most members of this flower-producing family are herbs while few numbers of shrubs and trees are also present. Moreover, *Asteraceae* has more than 40 economically important crops that fall in different categories depending on the crop type and economic specifications (Bessada et al., 2015). Among them, a few most significant food-crops include: *Lactuca sativa* (lettuce), *Cichorium intybus* (chicory), *Taraxacum* spp (dandelion), *Cynara scolymus* (artichoke), *Smallanthus sonchifolius* (yacón/ sweet carrot), *Tragopogon porrifolius* (salsify/ vegetable oyster), *Cynara cardunculus* (cardoon), and *Arctium* spp (burdock). Others include oil producing plants, such as *Helianthus annuus* (sunflower), *Carthamus tinctorius* (safflower); as well as herbs and aromatic plants particularly essential oils, that have shown incredible antimicrobial and antioxidant properties, such as: *Anthemis* spp (chamomile), *Echinacea* spp (coneflower), *Taraxacum* spp (dandelion), *Cichorium intybus* (chicory), *Calendula* spp (marigold), *Stevia rebaudiana* (stevia), and *Artemisia* spp (tarragon or wormwood) (Kenny et al., 2014). They produce several secondary metabolites such as alkaloids, pentacyclic triterpenes, acetylenes, tannins, organic acids and sesquiterpenes (Gallon et al., 2018). Some plant species have been used for producing nectars that belongs to the genera of *Centaurea* (safflower), *Helianthus* (sunflower), and *Solidago* (goldenrods). Moreover, numerous Asteraceous species have been utilized for ornamental purposes that includes most commonly *Dahlia* spp., *Chrysanthemum* spp., *Tagetes* spp. (marigold), *Achillea* spp. (yarrow), *daisy* spp., *Callistephus chinensis* (china aster), *Plectocephalus americanus* (basket-flower), etc. (Encyclopædia Britannica, 2020). These crops are globally distributed even though some does not belong to the temperate and Mediterranean zone, higher elevation and tropical regions (Mandel et al., 2017).

Despite having diverse medicinal properties, fungal pathogens are a common problem to Asteraceae worldwide. Especially among leafy vegetables, the most predominant category of edible greens including lettuce, that have production based under greenhouse or in semi-fields all across the Europe and other continents of the world (Alcon et al., 2010). Some of the most economically important fungal diseases along with their fungal causal agents are: Bottom rot (*Rhizoctonia solani*), Alternaria leaf spot (*Alternaria sonchi*), Fusarium wilt (*Fusarium oxysporum* f. sp. *lactucae*), Lettuce drop (*Sclerotinia sclerotiorum*,), Verticillium

wilt (*Verticillium dahliae*), Damping-off (*Pythium ultimum*), Gray mold (*Botrytis cinerea*), Septoria leaf spot (*Septoria lactucae*), Downy mildew (*Bremia lactucae*), Anthracnose (*Microdochium panattonianum*), Powdery mildew (*Golovinomyces cichoracearum*), and so on (Raid, 2004). Besides infecting lettuce, several genera of pathogenic fungi; including *Rhizoctonia, Fusarium, Verticillium, Pythium, Sclerotina, Phytophthora* and *Botrytis;* have threatened annual production globally by causing several diseases in major crops including cotton, wheat, vegetables, fruits, wheat and ornamentals with the approximate rate of 50%-70% yield loss (F. Aggeli et al., 2020; Baysal-Gurel and Kabir, 2018; Mihajlović et al., 2017).

The present work focused on the following fungi that can infect Asteraceae plants: *Rhizoctonia solani* Kühn*, Pythium ultimum*, and *Botrytis cinerea.*

Rhizoctonia solani kühn (Basidiomycota)

Rhizoctonia Solani J. G. Kühn (syn. *Thanatophorus cucumeris*, family: *Ceratobasidiaceae*) is a soil inhabiting plant pathogenic fungus that causes diseases in more than 200 plant species belonging to economically important crops by primarily attacking the roots, lower stems, corms, tubers, or underground plant parts (Yang and Li, 2012). *R. solani* causing bottom rot followed by damping off of seedlings is often recurrent in most countries, particularly Italy, where lettuce is grown throughout the year (Barrière et al., 2014; Garibaldi et al., 2006; Gullino et al., 2019; Subbarao et al., 2017). Besides lettuce, it also infects escarole and endive with bottom rot disease. On the other hand, *R. solani* isolates disrupt the global agricultural economy by causing black scurf of potatoes (Woodhall et al., 2008), bare patches in cereals (MacNish and Neate, 1996), damping off in soybean (Fenille et al., 2002), root rotting of sugar beet (Strausbaugh et al., 2011), belly rotting of cucumber (Erper et al., 2000), banded leaf and sheath blight in maize variety (Ning Li et al., 2019), foot rot and damping off in tomatoes (Gondal et al., 2019), and sheath blight of rice (Jamali et al., 2020).

This fungus exhibits relatively diverse morphology, physiology, host specificity and pathogenicity (Taheri and Tarighi, 2011). *R. solani* is made up of septate, white to brown hyphae that typically branch at near-right angle. It lacks sporulation and asexual fruiting structures. As a capable soil saprophyte, *R. solani* persists in crop debris in the form of sclerotia and mycelium where it disseminates across long distances via mechanical contact with contaminated soil, plant material or equipment. It penetrates directly and colonizes the plant parts both inter- and intracellularly. Its mycelial growth requires optimal temperature of around 25-30 °C and short interval of 36-48 hours to display initial symptoms upon infection. The biological specialization of *R. solani* isolates exhibits hyphal fusion (anastomosis) belonging to at least 13 anastomosis groups (AGs) based on their morphological, genetic, host specific, and pathogenic characteristics (Ajayi-Oyetunde and Bradley, 2018; Taheri and Tarighi, 2011). Based on the feeding strategies, *R. solani* exhibits its virulence through biotrophy, hemi-biotrophy and necrotrophy stages for their survival (Glazebrook, 2005; Horbach et al., 2011; Kouzai et al., 2018). Usually, it causes pre-emergence death of seedlings as well as

necrosis of the stem at or near the soil surface that can be marked distinctly between infected and healthy tissue.

Pythium ultimum Trow (Oomycota)

Pythium ultimum Trow (family: *Pythiaceae*) is well-known as a saprotrophic phytopathogen that produces oospores and survives in the soil debris for long periods, infecting roots and seeds of host plant which leads ultimately to the symptoms of necrosis and damping off (Kamoun et al., 2015). Its presence has been found in Australia, Japan, South Africa, Canada, Brazil, UK, USA, Europe and many other countries of the world (Gullino et al., 2019). Damping off, solely, has been progressively detected at pre-emergence as well as post- emergence stage among the species of family Asteraceae such as lettuce, wild rocket and lamb's lettuce (Matić et al., 2019). Moreover, *P. ultimum* has shown wide range of distribution in the field, experimental stations or greenhouses, affecting the yield and quality of agricultural products including wheat, canola, soybean, rye and maize, resulting in economic losses (Nana Li et al., 2019). *P. ultimum* show great survival capacities even under adverse conditions as thick-walled oospores. These oospores can survive for a long time and, when the conditions are suitable for infection, can germinate, forming sporangia and differentiating zoospore forming bodies. Zoospores, then move in the soil containing water, which then move towards root exudates and germinating seeds via chemotaxis. This homothallic oomycete is able to reproduce asexually by forming asexual sporangia or hyphal swellings to initiate the infection (Schroeder et al., 2013). Upon infection, *P. ultimum* causes deterioration of seed germination during the emergence of radicle or, should the seed manage to emerge, the hypocotyl penetration may occur which leads to the collapse of the plant subject to root-tip necrosis, stunted growth, malformation and consequent death (Subbarao et al., 2017). After the host is infected, symptoms other than damping off, such as water-soak root rot, leaf blight, crown rot, stunted growth could occur that results in host death eventually (Lin et al., 2018).

Botrytis cinerea Pers (Ascomycota)

Botrytis cinerea Pers (BC, family *Sclerotiniaceae*) emerged as a filamentous ascomycete phytopathogen infecting more than 1400 host plant species belonging to 586 genera of tracheophytes all around the world (Elad et al., 2016). Among these hosts are crop plants with great economic value, such as lettuce, tomatoes and petunia, grapes, rose, broccoli etc., which have shown huge loses at both pre- and post-harvest stages (Elad, 2016). Among the top ten, *B. cinerea* is considered as the second most invasive fungal phytopathogen globally, therefore, many studies have been conducted on the control of this pathogen (Dean et al., 2012). It persists mostly in the colder temperate zones, ranging from tropical and subtropical areas where vegetation occurs (Elad, 2016). *B. cinerea* produces gray to brown colored conidiophores at maturity that bears grapelike clusters of conidia at branch tips. The pathogen can enter through wounds but can also directly

penetrate the host plant. The necrotrophic nature of this pathogen makes its survival simpler in soil debris as a saprophyte where the conidia become windborne and land on senescent damaged tissue of pre-moistened host plant. The gray mold disease proceeds with water-soaked lesions followed by gray to brown mushy rot that develops first on the oldest leaves close to the ground and then formation of black sclerotia on infected tissues. These symptoms strike the seeds, leaves, fruits, buds and flowers of most crop plant species. Other symptoms can include stem canker/rot, leaf spots, blight, and damping-off of young seedlings being varied depending upon the crop genotype and other environmental conditions (Pikovskyi et al., 2018).

1.3. Chemical control

To overcome the apprehension of crop protection from phytopathogens and maintain high levels of production, chemical fertilizers and pesticides have been considered as the possible and efficient solution to control the diffusion of diseases, either by targeting the pathogen directly or by affecting the pests that acts as vectors for the diseases (Lamichhane et al., 2018). Since the mid-1900s, the implementation of chemical-based compounds in the field, during storage, transportation and marketing as formulated products, either synthetics (such as: cyprodinil, mancozeb, thiram) or natural inorganics (such as: arsenic, copper sulfate, lime sulphur), have been rationally used to contribute to crop protection and crop production. Even though the chemical-based strategies showed great efficiency, with high demand of chemical pesticides production of about 3 billion tons each year around the world, the agriculture economy is facing the global crop yield loss of about 40% annually (Messing and Brodeur, 2018). Not only that, some complications became more and more evident, particularly, the overuse, improper use and dependence on chemical fertilizers and pesticides have led to several concerns such as environmental and health-related risks, prevalence of widespread resistance against applied chemicals and subsidence of non-target beneficial microbial community (Cagliari et al., 2019). Such concerns reflected in the directives 128/2009 of the European Community that pointed at reducing the use of pesticides to the minimum and reinforced laws in finding sustainable alternatives to supplement the use of agrochemicals and less environment friendly practices.

1.4. Biological control

Among the alternative solutions, one that is surely of great importance for its applications at pre- and post-harvest and fascinating for scientists is biological control, often shortened as biocontrol. Smith (1919) stemmed the broader phenomenon of natural control as “Biological control” that recognizes natural ecosystem responsible for exerting pressure on populations through abiotic and biotic factors. This concept was further refined by DeBach (1964), by explicitly referring to biotic factors causing detrimental effects to a particular species. Biocontrol aims to reduce the population of target organisms by utilizing the potential natural enemies such as parasites, competitors, predators, pathogens or its toxins. In terms of plant pathology, Biological

control is the purposeful utilization of beneficial microbes (antagonists of pest or pathogen), or its molecules to suppress phytopathogens' ability to colonize the host or induce symptoms that causes the plant diseases (Heimpel and Mills, 2017).

By implementing biocontrol practices, the escalating worries over the use of pesticides can be reduced which is today's primary interest of agricultural industry. As it merely depends on natural approaches such as biopesticides, biofertilizers and crop residue return (Sayyed, 2019). Over the past decades, biocontrol practices have been indeed superordinated fourfold as compared to chemical control (Van Driesche et al., 2010). However, value-added collaboration between practitioners and scientists could implement cost-effective biocontrol research that obviously require exposition as compared to emerging control measures (Barratt et al., 2018).

1.4.1. Chitosan (Organic Resistance Inducers)

Chitosan is a deacetylated derivative of chitin, an amino-polysaccharide, found naturally in abundance in the biosphere and can be extracted in the form of biomass and food waste from the exoskeletons of insect or crustaceans, squid pen and fungal cell walls (Mujtaba et al., 2020). Chitosan as an organic resistance inducer, have emerged as a potential significant polysaccharide for various applications in the field of agriculture, medicine and cosmetics (Kurita, 2006). They are made available in biocontrol markets in the form of either a liquid solution or a water-soluble powder (Merino et al., 2018). Besides vast utilization of chitosan or chitin as a product, a broad variety of other biopolymers have been successfully applied as nanocarriers for the controlled delivery of agrochemicals, including cellulose, starch, collagen, and alginate (Choudhary et al., 2019; Wani et al., 2019)**.** Nevertheless, in plants, chitosan has gained ample attention as one of the most useful agrochemical carriers, thanks to its diverse characteristics incorporating biocompatibility, biodegradability, high permeability, cost-effectiveness, non-toxicity and an excellent film forming capabilities (Kashyap et al., 2015; Malerba and Cerana, 2019). Chitosan and its derivatives are being employed for the targeted delivery of toxins, plant hormones, herbicides, macro and micronutrients, either alone or in composite form (Cota-Arriola et al., 2013).

Chitosan is referred to as a plant elicitor locally and systemically that boosts the synthesis of secondary metabolites (Karuppusamy, 2009), notably polyphenols (Yin et al., 2012). Chitosan has antimicrobial properties and can simultaneously increase the growth of beneficial microbes in the plant parts, rhizosphere and soil (Ramírez et al., 2010). Consequently, several reports have been confirmed the higher crop yield production after treatment with chitosan products (Sharp, 2013). The antimicrobial activity of chitosan against phytopathogens (fungi, bacteria and viruses) is based on several mechanisms. (1) positive charges of the protonated chitosan activate the electrostatic interactions with the negative charge of the pathogen surface; (2) the cell damage and leakage of the pathogen causes subsequent escalation in the pathogen cell membrane

permeability and cell death; (3) chitosan pertains the chelating compounds (such as minerals, metal ions and nutrients) that causes inhibition of the pathogen's essential growth elements, resulting in an abnormal growth of the pathogen; (4) the penetrated chitosan binds with the nucleic acid machinery (including DNA/ RNA) of the pathogen, that prevents the mRNA production and pathogen reproduction in the plant cell; and lastly, (5) the deposition of chitosan on the microbial surface of phytopathogen generates a barrier that blocks the extracellular transport of effectors molecules, metabolites and essential nutrients from entering into the host cell, thereby, disrupting the pathogens growth (Liang et al., 2018; Xing et al., 2015).

1.4.2. dsRNA potential as pesticide (RNAi based biocontrol strategy)

Another strategy that can achieve pathogen control with reduced environmental impact and concerns is RNA interference (RNAi) via dsRNA, a natural product and occurs in most higher organisms in nature. It triggers RNA silencing, also known as RNA interference (RNAi), which is a conserved eukaryotic mechanism playing a crucial role in growth, development, host defense against viruses, and transposon inactivation, across plant, fungi and animal kingdoms. It is a newly identified mechanism of post-transcriptional regulation of gene expression which is highly conserved among high eukaryotes (Carthew and Sontheimer, 2009). It is a process in which dsRNA triggers the silencing of the complementary messenger RNA (mRNA) (Zamore, 2001).

RNAi is a naturally occurring cellular defense system mediated by double-stranded RNA (dsRNA). In most eukaryotes, long dsRNA found within a cell is seen as either a source of viral infection or as evidence of transposon activity, both of which the cell will seek to suppress (Obbard et al., 2009). In different organisms, the RNAi pathways comprise different proteins and mechanisms, but they operate by strikingly convergent strategies. In all the organisms that have been studied, RNAi involves two main components: small interfering RNA (siRNA) that determine the specificity of the response and Agronaute proteins (AGO) that carry out the repression. During their infection cycles in plant hosts, both DNA and RNA viruses produce dsRNA structures. In RNA viruses, dsRNA intermediates are formed during replication while in DNA viruses overlapping transcripts from opposite strands are generated.

Viruses are the inducers as well as targets of RNAi machinery. The overall process of RNAi-mediated gene silencing can be divided into three basic steps (Siomi and Siomi, 2009), as illustrated in Figure 3. First, a long dsRNA expressed or introduced into the cell is processed into small RNA duplexes (typically 19-21 nt long) by a RNase III enzyme known as Dicer forming what are called short interfering RNAs (siRNAs). Second, the small RNA duplexes are unwound and one of the strands, known as the guide strand, is preferentially loaded into the ribonucleoprotein complexes known as RNA induced silencing complexes

(RISC) containing, among others, the Argonaute proteins. Third, the RISC, directed by the guide strand, actively searches target RNAs containing specific nucleotide sequences complementary to the guide RNA and direct gene silencing by endonuclease cleavage of siRNAs (Carthew and Sontheimer, 2009). siRNAs complementary to promoter regions of target genes induce transcriptional gene silencing (TGS) leading to methylation of promoter and consequently inhibition of transcription. siRNAs complementary to mRNAs induce post-transcriptional gene silencing (PTGS), which results in sequence-specific RNA degradation. Both PTGS and TGS are correlated, but TGS functions in nucleus while PTGS occurs in cytoplasm (Vaucheret et al., 2001).

Therefore, RNAi is a sequence-specific method of suppressing a targeted gene's expression, and because each species is defined by the uniqueness of its genes' sequences, RNAi can potentially be designed in a species- specific, or even strain-specific, manner. By targeting genes essential for pathogen's growth, development, or reproduction, RNAi could be used selectively to control pathogens without adversely affecting non-target species (Whyard et al., 2009).

RNAi-derived resistance against viral diseases have been successfully employed to develop transgenic plants that are genetically modified with desirable traits, in this case the production of dsRNA molecules that match with the sequences of the target pathogen, making the plant preventively able to silence the genes of the pathogen (Jia et al., 2017; Patil et al., 2011). Besides the success and durability, the adoption and cultivation of transgenic crops pose certain worries regarding environmental hazards and potential health risks (Fermín et al., 2011; Fuchs and Gonsalves, 2007; Hamim et al., 2018; Ita et al., 2020). In this context, plant biotechnologists are exploring non-transgenic biotechnological interventions that can induce robust viral resistance to control plant viral diseases. Similar to the transgenic RNAi-derived virus resistance, non-transgenic RNAi-derived viral resistance can also be readily induced through topical/foliar application of dsRNA molecules in a highly specific manner (Das and Sherif, 2020; Holeva et al., 2006; Konakalla et al., 2019; Shen et al., 2014; Tenllado and Dıaz-Ruız, 2001; Vadlamudi et al., 2020; Voloudakis et al., 2015; Yan et al., 2020). DsRNA is the key molecule in the induction of RNAi-derived resistance in naturally-infected plants as well as in non-transgenic virus resistance when dsRNA is applied as a 'spray-on' technology (Das and Sherif, 2020; Jadhav et al., 2019; Liu et al., 2008; Waterhouse et al., 1998). Several reports were published for such dsRNA-mediated protection against different types of plant viruses such as: *Cucumber mosaic virus* (CMV) (Borah et al., 2018; Holeva et al., 2006), *Tobacco mosaic virus* (TMV) (Konakalla et al., 2016), *Zucchini yellow mosaic virus* (ZYMV) (Kaldis et al., 2018), *Bean common mosaic virus* (BCMV) (Worrall et al., 2019), CMV and *Tomato leaf curl virus* (ToLCV) (Namgial et al., 2019), *Sesbania mosaic virus* (SeMV) (Konakalla et al., 2019), *Papaya ring spot virus* (Shen et al., 2014; Vadlamudi et al., 2020) and many others.

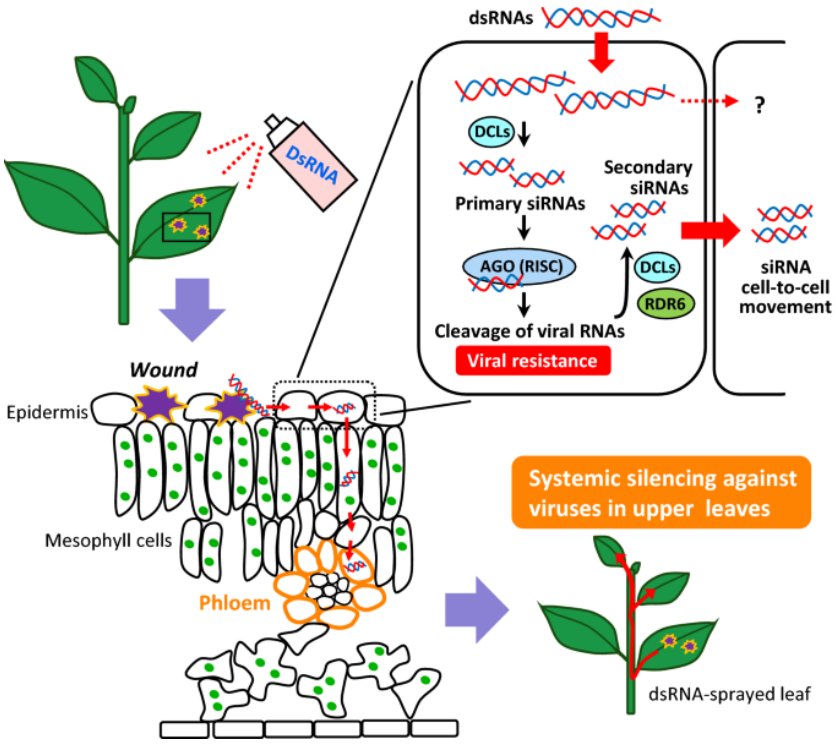

Figure 2. Application of exogenous viral RNAs on leaves to induce RNA silencing. *Sprayed dsRNAs will penetrate cells through a wound and be cleaved into siRNAs by cellular dicer-like proteins (DCLs). Generated siRNAs will then be incorporated into an RNA-inducing silencing complex (RISC) and function to guide argonaute protein (AGO) to cleave target viral RNAs. The secondary siRNAs amplified by RNA-dependent RNA polymerase 6 (RDR6) and DCLs will then move through the phloem to induce systemic silencing in upper, unsprayed leaves* (Kim et al., 2019).

Coat Protein

Based on dsRNA mediated resistance against plant pathogenic viruses, several viral genes and sequences were applied to many transgenic and non-transgenic plant species.

One of the most used target genes to produce virus resistant plants is coat protein (CP). Appreciable resistance level against virus infection has been reported in several transformed plants. A highly unpredictable resistance mechanism was reported on employment of CP gene-mediated defence against tospoviruses. When CP was used against similar isolates of tospoviruses it was RNA driven while in case of distant one, it was protein derived resistance (Pang et al., 2000). In transgenic potato plants CP gene of *Potato mosaic virus* (PMV) strain N605 reported to provide resistance against both the N605 and 0803 strains (Malnoë et al., 1994). Likewise, transgenic tobacco plants expressing a CP gene of TMV found to be resistant to TMV and

other closely related tobamoviruses such as: *Tomato Mosaic Virus* (ToMV), *Pepper Mild Mottle Virus* (PMMV), *Tobacco Mild Green Mosaic Virus* (TMGMV), *Ontoglassum Ringspot virus* (ORSV), *Ribgrass mosaic Virus* (RMV) and *Sunn Hemp Virus* (SHMV) (Beachy et al., 1990). While on the contrary, transgenic papaya expressing CP gene of *Papaya ringspot virus* (PRSV) strain HA found to be resistant only for HA strain of PRSV (Tennant et al., 1994). However, in most recent studies, Nemes and colleagues (2019) evidenced that the symptoms recovery is affected by the phosphorylation of CMV coat protein in tobacco plants. Moreover, the mutants of TMV coat proteins T42 and E50Q have exhibited more resistance as compared to WT coat protein, which confirmed as association with coat-protein mediated resistance against TMV (Sharma et al., 2020).

Remarkably, in contrast to transgene derived coat protein resistance, numerous studies confer viral resistance using non-transgenic approach derived from homologous viral genes, particularly, the CP derived dsRNA mediated resistance. As an example, the studies conducted by Konakalla and colleagues (2016) showed that the exogenously applied dsRNA molecules derived from the CP of TMV induced 50% resistance in *Nicotiana tabacum* cv. Xanthi plants against TMV. In a most recent study conducted by Vadlamudi and colleagues (2020), the CP derived dsRNA molecules of *Papaya ringspot virus* (PRSV) Tirupati isolate provided 100% resistance against PRSV-Tirupati infected papaya plants and 94% resistance against PRSV-Delhi infected papaya plants.

So, as per these findings it could be concluded that viral coat protein mediates either broad or narrow resistance in both transgenic and non-transgenic plants. Besides configuring coat protein mediated resistance, several other viral proteins have also been used to engineer resistance against viruses, like movement protein (Konakalla et al., 2019; Sijen et al., 1996), replicase-associated protein (Canto and Palukaitis, 1999; Chellappan et al., 2004; Johnson et al., n.d.), and the potyvirus susceptibility S-genes (Mäkinen, 2020).

Resistance Employing Transient RNA Silencing

The rising biosafety concerns regarding transgenic approaches have led towards the design of transient RNA silencing. In this technique, silencing molecules of RNAs are directly transferred into plant tissues. This strategy is implemented in plants through two modes: (a) mechanical inoculation, *in vitro* synthesized dsRNA molecules are injected into the plant and (b) *Agrobacterium*-mediated, where transient expression of dsRNA is brought about. dsRNA molecules are synthesized specifically according to the target so efficient resistance was reported against homologous virus species (Tenllado and Dıaz-Ruız, 2001). However, the immunity attained against the viral infection was non heritable and did not confer long-term protection (Tenllado et al., 2003). This methodology is very cost and labor intensive so it could not be used at field level. To overcome

this issue, dsRNA were synthesized in bacteria and crude extract of bacteria containing dsRNA was sprayed over the plant (Gan et al., 2010; Ita et al., 2020; Song and Ryu, 2017).

1.4.3. Beneficial microbes as Biocontrol Agents

To date, the utilization of microbial inoculants that show either plant growth promotion capabilities or biocontrol efficacy and sometimes both abilities, usually known as plant growth promoting microbes (PGPM) and biocontrol agents (BCAs), and have acquired particular importance in agriculture in response to the widespread usage of pesticides and fertilizers. In accordance with the global industry report 2019-2025, the beneficial microbes as biocontrol agents market, that showed the increment of 3 billion USD in 2018, has been now expected to surpass 15% Compound Annual Growth Rate (CAGR) by 2025 (Pulidindi Kiran and Pandey Hemant, 2019). The reasons for which the trend has shifted towards bio-based products are mainly due to their peculiar characteristics when compared to chemical pesticides and fertilizers. They are safer, display minimal environmental harm, demonstrate more targeted operation, have rapid decomposition processes, capable of multiplying but are also regulated by plants and indigenous microbes, successful in smaller amounts, less liable to exacerbate pathogens and pest resistance, and may potentially be used in both organic and conventional agriculture (Berg, 2009; Köhl et al., 2019).

Among various plant growth promoting fungal BCAs, the strains belonging to the genus *Trichoderma* is recognized worldwide as an appropriate example that have been considered as model organism to study plant-microbe interactions (Vinale and Sivasithamparam, 2020). It is estimated that around 90% of fungal strains that have been used as biocontrol agents came from various *Trichoderma* species (Hermosa et al., 2012). Furthermore, 60% of those species have been utilized to produce bio-fungicides that have shown great potential against pathogens (Abbey et al., 2019). Few examples include: *T. viride*, *T. atroviride, T. polysporum, T. harzianum, T. gamsii, T. asperellum, etc.* This imperfect fungus is rapid colonizer, iron-chelator, filamentous, invasive, usually avirulent, opportunistic and exhibit symbiotic relationship with the plants which makes it more favorable microbe in agro-industry (Woo et al., 2014). However, in a most recent study conducted by Kumar and Mukherjee (2020) provided an unusual insight regarding the strain *T. virens* to have been acting as maize pathogen. The Alt a1 protein of *T. virens* showed suppressive effect on the plant defense responses by downregulating the thaumatin-like protein PR5 gene in order to colonize the roots. A further study will be needed to interrogate this unusual virulent behavior of this strain. As in general, *Trichoderma* spp. interacts with the pathogen, directly through the hyper-parasitism, antibiosis and competing for nutrient and space as well as indirectly by promoting the plant growth, nutrient uptake, bioremediation of diseased rhizosphere and enhancing stress tolerance by activating secondary metabolites, enzymes and resistance proteins that leads to mediate induce systemic resistance as well as systemic acquired resistance (Kumar, 2013; Wilson et al., 2008; Zhang et al., 2017).

1.4.2.1. Endophytic Plant Growth Promoting Bacteria (ePGPB)

Bacterial endophytes commonly resides within the host plant tissue and colonize both the intracellular and intercellular spaces, while some of them resides in endosphere as well as in rhizosphere that is the characteristics of plant-growth-promoting-rhizobacteria (PGPR) (Bacon and Hinton, 2007; Reinhold-Hurek and Hurek, 1998), hence termed as endophytic plant growth promoting bacteria (ePGPB) for the present thesis study. They are often referred to as plant endosymbionts as they tend to have favorable relationship for both itself and its host plant, via plant growth promotion, plant defense induction or antimicrobial metabolites production (Hardoim et al., 2008). They are usually found in every plant part, including leaves (Tan et al., 2015), stem (Chung et al., 2015), root nodules (Martínez-Hidalgo et al., 2015), roots (Chen et al., 2019), seeds (Gond et al., 2015), and rhizomes (Jasim et al., 2014). The common example of ePGPBs belongs to the genera of *Pseudomonas, Microbacterium, Pantoea, Bacillus, Micrococcus, Stenotrophomonas, and Burkholderia* (Aswani et al., 2020; Shi et al., 2014; Sun et al., 2008).

Plant-ePGPB colonization patterns

The rhizosphere and phyllosphere are typically the host colonization source of endophytes. However, some are carried on by seeds. They are classified as facultative, obligate and passive endophytes based on their adaptive colonization capability (Khare et al., 2018). Facultative endophytes are the free-living bacteria present in soil microbiome, colonize host plant internally when opportunity arises via horizontal transmission manner and occurs through the rhizosphere following coordinated infection (Hardoim et al., 2008). The obligate endophytes depend on the metabolic activity of host plant for their survival and colonizes through seeds or through the action of vectors as a mean of vertical colonization and are not originated in the rhizosphere. Passive endophytes colonize the plant through open wounds along with root hairs and are often called associative bacteria due to their adherence to the root surface. However, this passive lifestyle of endophytes makes them less competitive since they lack cellular machinery required for colonization, hence, may be less suitable for plant growth promoters (Hardoim et al., 2008).For a successful colonization of the host, facultative ePGPB interacts with the host plant via chemotaxis mediated by root exudates, adherence to the plant root surface through quorum-sensing signaling and biofilm formation, and penetration in the internal parts of plants through the secretion of cell wall degrading enzymes (Kandel et al., 2017). On the other hand, plants do molecular interaction with the bacterial endophytes through various receptors i.e. receptor-like kinases (RLK) and phytohormone signaling pathways as a symbiotic or defensive response. For a symbiotic response, the endophytic bacteria produce its own microbe associated molecular patterns (MAMPs), which is recognized by the specialized plant receptor RLK called as flagellin sensing 2 (FLS2) that functions as a PRR (a key component of innate plant immunity) and binds to flg22 of flagellin. This leads to a rapid extracellular alkalization, reactive oxygen species (ROS) production, mitogen-activated protein kinase (MAPK) cascade

activation, and upstream pathogenesis related (PR) protein regulation in the host plant (Vandenkoornhuyse et al., 2015). Whereas, as a defensive response, plants mediate phytohormones mediated by SA, ET and JA to regulate bacterial colonization and microbial diversity distribution (Pinski et al., 2019).

Direct and Indirect mechanisms employed by ePGPB

ePGPB are usually called endosymbionts and carry several genes including the defense related genes, which are advantageous for plants and modulate the growth of the host plant alongside providing tolerance against various stresses (Khare et al., 2018). They often act directly or indirectly and sometimes simultaneously, to promote host growth through the production of plant hormones, mobilization of nutrients, and lowering of ethylene-related stress in plants; and to fight against various phytopathogens by mechanisms such as competition for nutrients and niche (CNN), production of allelochemicals including antibiotics as antimicrobial substances, cell wall degrading enzymes, and induction of host systemic resistance (ISR) (Saad et al., 2020).

Among ePGPB, the following plant growth promotion is considered as a phenomenal indirect trait when the host is under biotic stress condition (Fig. 2A); (i) They stimulate the production of phytohormones that play a major role by benefitting the growth promotion and changes the morphology and structure of the plant. Such as auxins production (Dutta et al., 2014), ethylene (Kang et al., 2012), and gibberellic acid (Khan et al., 2014), by triggering plant cell division, differentiation and extension and stimulates seed germination and have functions in vascular system development, adventitious root formation, biomass formation, pigmentation, and abiotic stress resistance (Khan et al., 2014; Yadav and Yadav, 2017); (ii) mobilization of nutrients which is commonly known as biofertilization that includes: fixation of atmospheric nitrogen (Shcherbakov et al., 2013), solubilization of minerals i.e. phosphorus (Alori et al., 2017; Smyth, 2011) and formation of siderophores to scavenge iron ions (Yadav, 2018); (iii) lowering of plant stress hormones such as modulating the ethylene levels by an enzyme 1-aminocyclopropane-1-carboxylate deaminase. The process has been described in the studies of Hardoim, van Overbeek and van Elsas (2008), which confers the competency of several strains that enables interference with the physiology of host plant. Few discovered genera that have shown ACC deaminase production are *Agrobacterium, Enterobacter, Acinetobacter, Serratia, Alcaligenes, Bacillus, Ralstonia, Achromobacter, Rhizobium, Pseudomonas* and *Burkholderia* (Kang et al., 2012); and (iv) as rhizomediators, in which, they provide the protection of plants from the environmental pollutants in the form of bioremediation. A wide range of bacterial genera including *Bacillus, Stenotrophomonas, Sphingomonas, Burkholderia, Acinetobacter, Serratia* and mainly *Pseudomonas* have been involved in Polychlorinated biphenyls (PCBs), a type of persistent organic pollutants that are high risk synthetic substances against environment and human health (Sánchez-Pérez et al., 2020).

ePGPB utilizes this strong indirect mechanism of competition for ecological nutrients and niche (CNN), to prevent pathogens from colonizing the host tissue (Martinuz et al., 2012). Being able to colonize locally and systemically, ePGPB occupy the space and utilize nutrients, making them unavailable for pathogens to carry out their activities. This mechanism usually rely on other direct/ indirect mechanisms since most of its control methods is employed locally, however, for systemic colonization, the biocontrol efficacy depends on its competency of triggering responses (Fadiji and Babalola, 2020).

Antibiosis is the direct biocontrol phenomenon known for releasing allelochemicals in the form of antimicrobial secondary metabolites such as antibiotics and other volatile compounds produced by numerous endophytic bacteria to counteract the pathogenesis of disease producing microbes (Brader et al., 2014; Fravel, 1988). Iturin is a compound that have shown various antifungal properties by triggering ROS burst, disruption of cell-wall integrity and affected fungal signaling pathways (Han et al., 2015). Bacteriocins creates an imbalance in ions and solutes concentration by producing pores in the protective layers of targeted cells which leads to cell death of the pathogens (Subramanian and Smith, 2015). Furthermore, surfactin, fengycinin, phloroglucinols are well-known antibiotics produced by bacterial endophytes to inhibit the incidence of pathogen diseases. Some other examples of antibiotic compounds include 2, 4-diacetylphloroglucinol (DAPG), pyrrolnitrin, phenazine, tropolone, amphisin, oomycin A, hydrogen cyanide, tensin, and pyoluteorin. They are found in the form of either volatile organic compounds (VOCs) that are lipophilic molecules with low molecular weight or diffusible antibiotics (Beneduzi et al., 2012). These antibiotics interfere with the cell wall, cell membrane and cytoplasm inhibits their synthesis by direct mode of action (Liu et al., 2017; Maksimov et al., 2011).

Another direct mode of interaction is the production of cell wall degrading enzymes via lytic enzymes that have a function in the hydrolysis of polymers (Gao et al., 2010). ePGPB are capable of secreting enzymes like chitinases, hemicellulases, cellulases, 1,3 glucanases, pectinases, and so on. These enzymes may trigger an enhanced antagonistic activities along with the activation of other mechanisms (Babalola, 2007).

Induced systemic resistance (ISR) mediated by ePGPB is the most important aspect of indirect biocontrol that allow the host plants to better resist against phytopathogens (Junaid et al., 2013). Several bacterial endophytes have been reported to prime the plants (enhanced immune state), so that when the pathogen attacks, they can provide faster and more intense defense responses at low physiological cost to the plant. The ISR mediated by bacterial endophytes primes plant defense mechanism to combat wide range of future phytopathogens that functions as the mediator of Jasmonic acid (JA) and ethylene (ET) (Alvin et al., 2014) as well as salicylic acid (SA) (Sharma et al., 2017). The activity of ISR via bacterial endophytes usually involves interconnected signaling pathways of SA, JA and ET (Pieterse et al., 2012). These proteins exhibit functions such as lysis of pathogen cells, reinforcement of cell walls and programmed cell death. The most common ePGPB that are involved in the elicitation of defense priming by ISR are *Pseudomonas* spp, *Serratia*

spp. and *Bacillus* spp. The studies conducted by Niu and colleagues (2011) showed that *Bacillus cereus* AR156 strain triggered both JA/ET and SA signaling pathways in *Arabidopsis thaliana* mediated by ISR. Moreover, in another study, endophytic Actinobacteria showed an upregulation of JA/ET against phytopathogenic bacteria *Erwinia carotovora* and an upregulation of SA transcripts towards pathogenic fungus *Fusarium oxysporum* in *A. thaliana* (Conn et al., 2008). Similarly, the research of Brock and colleagues (2013) revealed that *Enterobacter radicincitans* DSM-16656 strain, an incredibly competitive colonizer of the endophytic ecosystem of different crops, is capable of inducing priming through SA- and JA / ET-dependent pathways in *A. thaliana* (Fig. 2B).

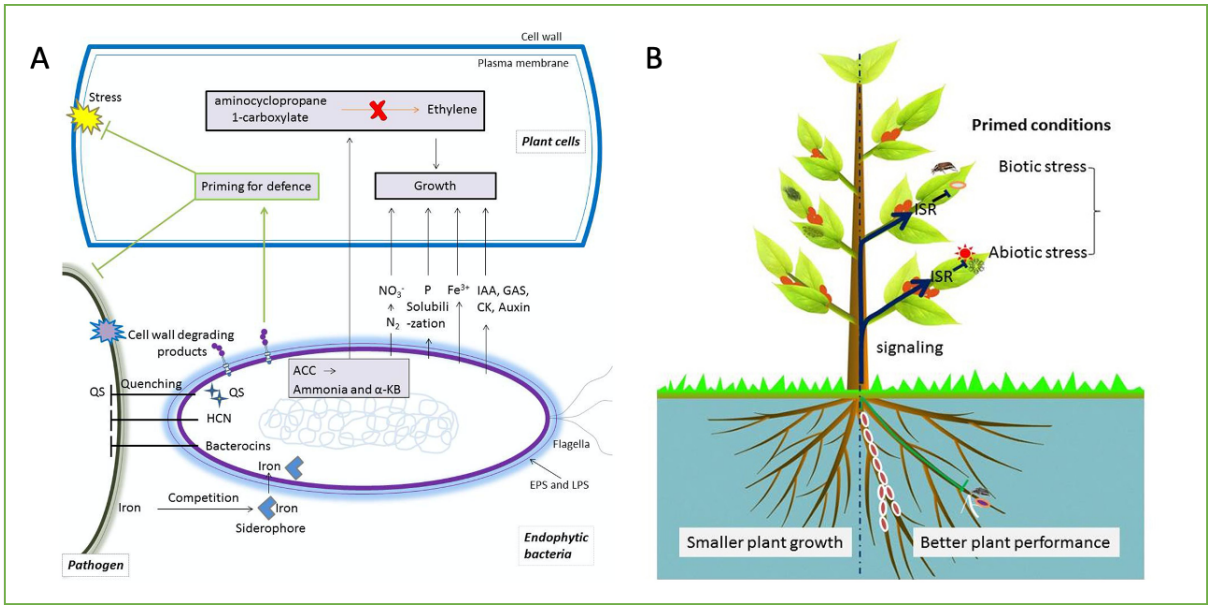

Figure 3. Schematic representation of endophytic plant growth promoting bacteria (ePGPB) summarizing plant growth promoting and biocontrol traits (Liu et al., 2017)

Role of ePGPB in plant microbiome

Plants harbor distinct microbial communities that are particular for each plant parts, such as: the phyllosphere (Vorholt, 2012), endosphere (Hardoim et al., 2015), and rhizosphere (Philippot et al., 2013). In agro-industry, plant microbiome is considered as the main contributing factor to the plant health and crop productivity (Berendsen et al., 2012). From the past decades, huge amount of evidences have illustrated the value of plant microbiome as a systemic booster of plant immune system through priming mediated by defense system (Van der Ent et al., 2009). Therefore, several studies centered on understanding the rhizosphere microbiome, as soil-derived microbes surrounds the root that is considered as potential source for selecting beneficial microbes impacting plant health in a positive way (Philippot et al., 2013). Moreover, understanding the microbiota is so important in determining the physiology, plant age, associated genes and its functions are essential for plant adaptation to different environments and changes (Bulgarelli et al., 2013).

Numerous studies have carried out to identify how the microbiome is influenced by phytopathogenic microorganisms (Hamonts et al., 2018; Larousse and Galiana, 2017). These studies explain the distinction between the taxa present in particular disease-affected plants and healthy plants, the identification of shifts in microbial populations that may be interesting indicators of plant health status, the description of healthy microbiome features, and even of specific strains or species peculiar to healthy plants.

It is a fact that the competitive ePGPB can migrate within the cellular compartments of plants and rhizosphere or soil which makes them diverse and most interesting to exploit for plant health (Maropola et al., 2015). Several corresponding literature have indicated that ePGPB can effectively colonize the host plant, and help in maintaining the whole microbiota of host in healthy condition along with direct beneficial effects on the host (Compant et al., 2010; Lugtenberg and Kamilova, 2009; Ryan et al., 2008). Recently, the studies conducted by Raza and colleagues (2020) demonstrated that VOCs derived from the three most common genera of ePGPB (*Bacillus*, *Paenibacillus* and *Pseudomonas*) was positively correlated with the pathogen suppression, however, the produced VOCs belonged to a diverse set of functioning. Thus, the implementation of competitive community rich ePGPB that are able to alter the physiology, promote plant growth and circumvent pathogen attack by triggering defense responses, can be employed for the agriculture improvement that ensure sustainability in crop protection and production.

1.5. Examined potentials of previously studied ePGPB strains

In the present study, five putative ePGPB strains were selected and examined:

- Strain 255-7, *Pantoea agglomerans*
- Strain 260-02, *Pseudomonas syringae*
- Strain S4C11, *Lysinibacillus fusiformis*
- Strain R8, *Paraburkholderia fungorum*
- Strain R16, *Paenibacillus pasadenensis*

As in the previously conducted studies, several ePGPB strains were isolated, characterized and tested for their plant growth promotion competency through several assays carried out at different levels (*in vitro*, *in vivo*, *in planta*) along with their biological control capabilities against various phytopathogens (mainly viruses, fungi and bacteria) on numerous model plants and host plants.

With a brief background, the strains S4C11, 255-7 and 260-02 were isolated from the roots of apple trees from an orchard belonging to the Minoprio Foundation, located in the town of Vertemate con Minoprio (CO), Italy, as part of a research on apple proliferation disease for two consecutive year of 2011 and 2012. Whereas, the strains R8 and R16 were isolated from leaves of recovered grapevine plants, surveying from the

phytoplasma associated with flavescence dorée disease in the years between 2006 and 2009, from a Barbera cultivar vineyard in the Oltrepò pavese area, in the province of Pavia, Italy (Bulgari et al., 2011). All these bacteria were either isolated from healthy plants or from plants that underwent recovery from the disease.

It was hypothized that these strains might have an effect on carrying out biocontrol activities since the host plants showed a particular behavior regarding these phytoplasmatic diseases. Therefore, the first study was conducted via *in vitro* biochemical assays (Phosphate solubilization, chitinase production, siderophore production, ACC-deaminase activity, catalase activity, IAA production,), that revealed several traits associated to plant-growth promoting rhizobacteria (PGPR) i.e. rhizosphere competence and endophytic lifestyle (Table 1).

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	**260-02**	**S4C11**	**R8**	**R16**
Siderophore production	-	+	+	+	-
Phosphate solubilization	+	+	-	+	-
ACC deamination	-	-	-	+	-
IAA production	+	+	+	+	+
Nitrogen fixation	-	-	+	+	-
Catalase activity	+	+	+	+	+
Chitinase activity	-	-	-	-	+
Motility	+	+	+	+	+

The second study was proceeded to assay these bacteria in a three-step approach by Passera (2017), starting from *in vitro* preliminary assays, moving to *in vivo* assays, and concluding with *in planta* assays. It was aimed to determine whether the selected strains could promote plant growth and, more importantly, control diseases caused by bacterial (*Pseudomonas syringae* pv. *tomato* DC3000 and integrated pUTgfp2x plasmid DC3000::*gfp* strain), fungal (*Aspergillus* sez. *nigri* AsN1 strain, B. *cinerea* Pers. MG53 strain, *Fusarium verticillioides* (Sacc.) Nirenberg, GV2245 strain, *Phomopsis viticola* (Sacc.) Sacc., PV1 strain, and *Rhizoctonia solani* (Cooke) Wint, RS1 strain), and viral pathogen *Cymbidium Ringspot Virus* (CymRSV). Following plants were grown as seeds that exhibits these pathogens as natural and model host plants (*Capsicum annuum*, var. Quadrato d'Asti; *C. annuum*, Zebo F1 hybrid; *Cucumis sativum*, Burpless Tasty Green F1 hybrid; *Cucurbita pepo*, var. Genovese; *Nicotiana benthamiana; N. tabacum; Phaseolus vulgaris*, var. Nano Bobis sel. Monica; *Solanum lycopersicum*, var. Rio grande; *S. lycopersicum*, var. San Marzano 2; and *S. lycopersicum*, Sibari F1 hybrid; *S. melongena*, Beatrice F1 hybrid; and *S. melongena*, Violetta lunga F1 hybrid). Furthermore, the detached plant parts of *S. lycopersicum* (cherry tomato berries) and *Vitis vinifera*, var. (Red Globe leaves) was also used throughout this study. The *In vitro* bioassays included dual-culture assays, dual plate assays particularly for the characterization of VOCs, cultural filtrate assay, and co-culturing technique), whereas, *In vivo* bioassays were conducted on detached tomato berries and grapevine leaves. *In planta* bioassays were performed based on pathogenicity tests, colonization assays, PGP ability assays, evaluation of methylation level, and biocontrol assays. In silico analysis was performed through Illumina HiSeq1000 for genome sequencing and assembly by using hybrid Illumina-minION approach for genome annotation and functional analysis as well as comparative pangenome analysis. The collective results obtained from the above-mentioned studies (particularly, biochemical test, genome analysis and *In planta* assays) are presented in Table 2.

Table 2. Table reporting an overview of examined endophytic plant growth promoting bacteria (ePGPB) extracted from the past studies based on biochemical, plant growth promotion and biocontrol traits **(Passera, 2017)**

	Pantoea agglomerans **(255-7)**	***Pseudomonas syringae*** **(260-02)**	***Lysinibacillus fusiformis*** **(S4C11)**	***Paraburkholderia fungorum*** **(R8)**	***Paenibacillus pasadenensis*** **(R16)**
Biochemical traits and the genes involved (Genome analysis extracted from Illumina and MinION)					
Genome size (Mbp)	4.753	6.052	5.007	9.172	5.727
Siderophore production	-	+	+	+	-
	19	18	36	3	**24**
IAA production	+	+	+	+	+
	4	4	5	5	7
Catalase activity (ROS detoxification)	+	+	+	+	+
	73	81	38	108	41
Chitinase activity	-	-	-	-	+ ***Farnesol**
	10	0	14	6	71
Motility (and Chemotaxis)	+	+	+	+	+
	74 (49)	89 (54)	82 (54)	96 (56)	21 (20)
PGP and Biocontrol traits (*In planta* bioassays)					
PGP (*pepper plants*)	+	+	+	+	+
Against Fungi	*B. cinerea* strain MG53 (Tomato)	*B. cinerea* strain MG53 (Tomato), *P. ultimum* and *R. solani* (Lettuce)	*B. cinerea*, *P. viticola*, and *R. solani (*Tomato, Lettuce*)*	*B. cinerea* strain MG53 (Tomato)	*P. viticola*, *B. cinerea* strain MG53 (Tomato), *P. ultimum* and *R. solani* (Lettuce)
Against Bacteria	*P. syringae* pv. *tomato* strain DC3000 (Pepper)	*P. syringae* pv. *tomato* strain DC3000 (Pepper, Tomato)	*P. syringae* pv. *tomato* strain DC3000 (Pepper)	*P. syringae* pv. *tomato* strain DC3000 (Pepper)	*P. syringae* pv. *tomato* strain DC3000 (Pepper, Tomato)
Against Viruses	*CymRSV* (Pepper)	*CymRSV* (Pepper)	*CymRSV* (Pepper)	*CymRSV* (Pepper)	*CymRSV* (Pepper)

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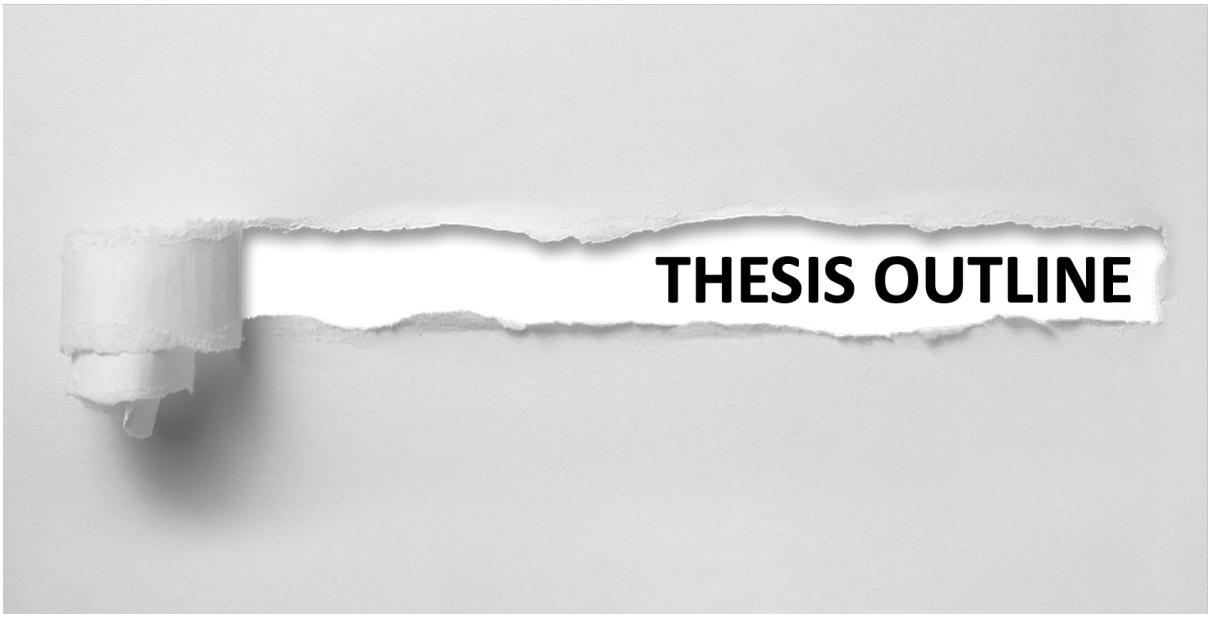
THESIS OUTLINE

Thesis outline

Based on the previous findings, I aimed to take a step forward and increase my understanding of model host plant *N. benthamiana* and natural host plant *Lactuca sativa* L. response mainly attributed to molecular interactions against two domains of phytopathogens i.e., viruses and fungi, respectively. The objectives of both actions were comprised in the form of various chapters as described briefly in the below-mentioned sections.

The first aim (**action against plant pathogenic viruses**) involved the following two chapters.

In Chapter 1, the experiments were conducted to evaluate the biocontrol ability of five endophytic plant growth promoting bacteria (ePGPB) strains; *Lysinibacillus fusiformis* (S4C11), *Paraburkholderia fungorum* (R8), *Paenibacillus pasadenensis* (R16), *Pantoea agglomerans* (255-7), and *Pseudomonas syringae* (260-02) in *N. benthamiana* plants infected with four phytopathogenic viruses; *Cymbidium ringspot virus* (CymRSV), *Cucumber mosaic virus* (CMV), *Potato virus X* (PVX), *Potato virus Y* (PVY), respectively. Their effects were also compared with three chitosan-based products which are known to induce resistance in plants. This evaluation was based on the observed phenotypic parameters of plant heights (cm), systemic infected leaves (%) and symptoms severity (%). Furthermore, the priming action of ePGPB strains were evaluated on healthy plants by the expression levels of defense-related genes *Enhanced Disease Susceptibility-1* (EDS1), *Non-expressor of Pathogenesis-related genes-1* (NPR1), and *Pathogenesis-related protein-2B* (PR2B), to have more clear understanding on the molecular basis behind the observed phenotype.

The Chapter 2 was directed to evaluate the potential of dsRNA molecules in the plants infected with homologous virus. For that, preliminary studies were conducted on the induction of resistance against *Tomato Aspermy Virus* (TAV) by exogenous application dsRNA molecules produced on the base of the gene encoding for the coat protein of the virus. The dsRNA molecules derived from coat protein gene of TAV was constructed by two-step PCR assays followed by *in vitro* transcription and purification. For the CP derived TAV dsRNA molecules and TAV inoculum, solution buffer containing TAV sap was diluted at the ratio of 1:5 and 1:10 for better understanding of the quantity inoculation in *N. benthamiana* plants. For this bioassay, only phenotypic parameters were evaluated based on four symptoms severity factors (mosaics and blisters, crinkling, leaf distortion, and systemic vein clearing) and plant heights (in regard of stunted growth of plants).

The second aim (**action against plant pathogenic fungi**) involved the following three chapters.

In Chapter 1, three ePGPB strains; *Paenibacillus pasadenensis* (R16), *Pseudomonas syringae* (260-02), and a commercial product *Bacillus amyloliquefaciens* (CC2); were evaluated first as biocontrol agents against *Rhizoctonia solani* and *Pythium ultimum* infected Romaine lettuce at pre-harvest stage and then their ecological impact on non-infected phytomicrobiome (sterile and non-sterile roots, rhizosphere and soil) was tested. Their biocontrol potential was also compared with the commercially available *Trichoderma* spp.-based product. It was followed by phenotypic parameter evaluation (based on severity of symptoms caused by both pathogens) and the percentage of viable seed germination assay. Furthermore, the microbiota analysis (based on bacterial communities) was conducted to elucidate the effect of these strains on the microbial diversity inside the root tissues.

The Chapter 2 was the continued phase of previous experiments of chapter 1, in which the abovementioned three ePGPB strains were further tested along with the other three strains *Lysinibacillus fusiformis* (S4C11), *Paraburkholderia fungorum* (R8), and *Pantoea agglomerans* (255-7). The first experiment was performed to test the biocontrol potential of these strains via % seed germination assay by using four different varieties of lettuce: *L. sativa acephala* (Lattuga Rosa), *L. sativa capitata* (Lattuga Meraviglia d'inverno), *L. sativa* L. var. *longifoglia* (Lattuga Romana) and *L. sativa capitata* (Lattuga la Resistente). The samples were evaluated after 5 days of post inoculation. Then, the second assay was conducted at 7 dpi using serial dilution and plating methods, to determine the direct and indirect antagonistic feature of three strains (R16, 255-7 and 260-02) against the pathogen in rhizosphere soil and soil grown under Lattuga Romana seedlings. The last objective of this chapter was to understand the molecular basis behind the observed direct/ indirect biocontrol effect of three strains (R16, 255-7 and 260-02). For that, we evaluated three salicylic acid (SA) mediated defense genes; *Pathogenesis-related protein 1* (PR1), *Phenylalanine Ammonia Lyase* (PAL), and *Thaumatin-like Protein 3* (ThlP3); and two ethylene/ jasmonic acid (ET/JA) mediated defense genes (*Ethylene Response Factor1* (ERF1), and *1-AminoCyclopropane-1-Carboxylate Synthase* (ACCS1); upon *R. solani* infected/ non-infected Lattuga Romana seedlings at 24 and 48 hours of post inoculation.

Finally, in Chapter 3, we extended our research on affirming two notions regarding the ePGPBs biocontrol potential of three strains (R16, 260-02, and CC2) in response to the chapter 1, whether these strains have the ability to control *Botrytis cinerea* strain MG53 infection in Romaine lettuce at post-harvest level and how they may impact the nutritional properties during

cultivation and shelf life. The biocontrol potential was monitored *in vitro* by using leaf material for 10 days of growing under controlled greenhouse conditions. The symptoms of infection were observed by storing the leave disks in 1 % agar-water plates and whole leaf part in humid chambers. Whereas, the parameters considered for the evaluation of the nutritional value included phenolic compounds, total anthocyanins, total carotenoids, vitamin C and antioxidant capacity measure at the time of harvest and at 1, 3, 6, and 8 days of storage.

In the last section, I concluded with a summarizing discussion on the outcomes of my PhD research and a reflection on the questions that remain for future research.


Aim# 1
Action against plant pathogenic viruses

1

ePGPBs priming action against phytopathogenic viruses and its molecular interaction with the host plant

The contents of this chapter are under the process of revision for journal publication

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Abstract

Plant viruses are a serious threat to crop systems that can be difficult to manage with traditional approaches, making the use of biocontrol a promising strategy. While it is known that many bacteria can induce systemic resistance in the plant, which is an effective mechanism against viral infection, more research is necessary to employ this strategy in agriculture. The present study aims to evaluate the antiviral effect of four endophytic plant growth promoting bacteria (ePGPB) strains (*Lysinibacillus fusiformis* strain S4C11, *Paraburkholderia fungorum* strain R8, *Paenibacillus pasadenensis* strain R16, *Pantoea agglomerans* strain 255-7, *Pseudomonas syringae* strain 260-02) against four viral pathogens (*Cymbidium ringspot virus* – CymRSV; *Cucumber mosaic virus* – CMV; *Potato virus X* – PVX; *Potato virus Y* – PVY) on *Nicotiana benthamiana* plants under controlled conditions, comparing their effects with those of three chitosan-based products, which are known to induce resistance in plants. Phenotypic evaluation of the plants showed that all bacterial treatments and one of the chitosan products managed to significantly reduce the symptoms caused by CymRSV, with a reduction up to 50% in symptom severity, and increasing plant height to be on par with non-infected controls. Four out of five bacterial strains reduced the severity of symptoms caused by CMV, with a reduction of up to 36%. The other two viruses were not found to be as efficient to biocontrol by these strains. Relative quantification of viruses and three defense-related plant genes (EDS1, NPR1, PR2b) showed that these phenotypic effects were unrelated to the concentration of virus in the plants and are accompanied by an overexpression of the EDS1 gene, suggesting the involvement of a salicylic acid-related defense pathway. Further studies will be needed to evaluate the duration of the protective effect and the epidemiological implications of the use of similar biocontrol strains, that reduce the symptoms but not the concentration of virus in the host.

Keywords: *Endophytes plant growth promoting bacteria (ePGPB), plant growth promotion, biocontrol, plant viruses, Induced systemic resistance (ISR).*

1. Introduction

Plant viruses are obligate pathogens responsible for nearly half of all known plant diseases and pose a major threat to agricultural production worldwide (Ziebell and Carr, 2010; Hadidi and Barba, 2011; Lewsey et al., 2018; Yu et al., 2019). According to the Ninth Report issued by The International Committee on Taxonomy of Viruses, there are 950 different plant viruses undermining plant health throughout the world (King et al., 2012), and new viruses are being discovered yearly. Plant viral infections leads to substantial losses in yield and fruit quality, thus adversely affecting human well-being due to agricultural and economic losses, which may have implications for biodiversity conservation (Souiri et al., 2020). The majority of these viruses have been described as RNA viruses (Soosaar et al., 2005), in particular single-stranded positive-sense RNA viruses (Dolja and koonin, 2011), and can cause up to 40% of the losses in important crops (Borah et al., 2013).

Unlike most other plant pathogens, viruses are difficult to control at the post-infection stage in the host plants (Hadidi and Barba, 2011). Therefore, the containment strategies against viruses are focused on preventing the infection in the first place (Sastry et al., 2014; Castle et al., 2017; Smith et al., 2019). The main techniques that can be employed to prevent infection are the control of vectors, reduction of inoculum sources, and the use of resistant plants. The use of pesticides against vectors has been the main protection strategy in the last years, but this approach can have a high cost and environmental impact (Pretty and Bharucha, 2014; Zhao et al., 2017), can be thwarted by the development of resistance to the pesticides (Luo et al., 2010), and can be ineffective against some virus-vector combinations, depending on their ecology and method of transmission. The reduction of the sources of inoculum is carried out mostly by employing certified virus-free plant material in fields (Laimer and Barba, 2011). The obtainment of resistant plants can be achieved through different methods, such as conventional breeding (Wang et al., 2011), or biotechnological approaches such as transgenesis or genome editing (Ziebell and Carr, 2010), but these approaches can take a long time, not be accepted in some countries, and may be outpaced by the evolution of the pathogen which, especially in the case of viruses, can mutate rapidly.

Other approaches that have been researched to pursue a sustainable management of viral diseases is the use of organic products, such as the inoculation of biocontrol agents or resistance inducers. Among organic resistance inducers, chitosan and its derivatives are surely the most well-known, and have been utilized as plant growth promoter and biocontrol molecule because of its biodegradability, biocompatibility and non-toxic nature (Bellich et al., 2016).

Chitosan is derived from the chitin in its deacetylated form present in the exoskeleton of crustaceans and considered as the second most abundant polysaccharide (Kanmani et al., 2017). Chitosan has been reported several times as being capable of triggering defense responses against plant viruses (Iriti and Faoro, 2008; Lu et al., 2010; Mishra et al., 2014; Firmansyah et al., 2017).

Whereas, the use of biocontrol agents provides similar benefits, as these microorganisms not only can protect the crops from pathogens, but often promote growth of the host plant through several mechanisms, such as providing nutrients (Glick, 2012), secreting allelochemicals and plant hormones or hormone-like substances (Bhore et al., 2010; Cipollini et al., 2012; Phetcharat and Duangpaeng, 2012; Rashid et al., 2012; Ullah et al., 2017). Considering the way to control viruses biologically, a specific category befalls, known as endophytic plant growth promoting bacteria (ePGPB), which have shown the ability to act against a broad spectrum of viral diseases, including *Cucumber mosaic virus*, *Tomato spotted wilt virus*, *Banana bunchy top virus*, *Tobacco mosaic virus,* and so on (Raupach et al., 1996; Zehnder et al., 2000; Murphy et al., 2003; Kandan et al., 2005 and Kavino et al., 2007, Wang et al., 2009, Yang et al., 2012, Kumar et al., 2016, Beris et al., 2018). As a recent example, the study conducted by Gupta and colleagues (2019) demonstrated that the application of *Pantoea agglomerans* strain UN1 24 hours prior to the inoculation of *Tobacco mosaic virus*, developed a systemic antiviral resistance and growth promotion of *Nicotiana glutinosa* and *Nicotiana tabacum cv. Xanthi-nc* plants. The results obtained this far indicate that these bacteria do not only act directly against the virus but also provide an indirect antagonism by stimulating induced systemic resistance (ISR) response in the host (Pieterse et al., 2014). ISR leads towards the activation of several defense-related genes through the jasmonic acid, ethylene and salicylic acid-mediated pathways (Wang et al., 2009; Alazem et al., 2015; Li et al., 2016), providing protection against a broad range of pests and pathogens, including viruses.

In this scope, the present work aims at characterizing the priming efficacy and mechanism of action of selected candidate ePGPBs against plant viruses and comparing their effect to that of chitosan-based products, in the model host plant *Nicotiana benthamiana*. The selected bacterial strains are *Lysinibacillus fusiformis* strain S4C11, *Paraburkholderia fungorum* strain R8, *Pantoea agglomerans* strain 255-7, *Paenibacillus pasadenensis* strain R16 and *Pseudomonas syringae* strain 260-02, the latter two having already been reported as plant-growth promoting and biocontrol agents against different pathogens (Passera et al., 2017; Passera et al., 2019; Passera et al., 2020).

ePGPB efficacy was tested on four utmost potent plant viruses, named as: *Cymbidium ringspot virus* (CymRSV, family *Tombusviridae*), *Cucumber mosaic virus* (CMV, family *Bromoviridae*), *Potato virus X* (PVX, family *Alphaflexiviridae*), and *Potato virus Y* (PVY, family *Potyviridae*). All of the above-mentioned phytopathogenic viruses belong to the genome of single-stranded positive-sense RNAs, which are renowned to cause diseases in major crop plants of different families, particularly, on the third largest and economically important family, a *Solanaceae* (Hollings et al., 1977; Douine et al., 1979; Brunt et al., 1996; Yardimci and Eryigit, 2006). CymRSV (monopartite) is a virus with no known vectors and rarely occurs as a major threat to agriculture, whereas, the other three viruses; CMV (tripartite), PVX (monopartite) and PVY (monopartite); have been known to cause severe damage with a broad host group (Edwardson and Christie, 1991; CABI, 2020; Miozzi et al., 2020) and vector range (Palukaitis and García-Arenal, 2003). The common symptoms include leaf crinkling, mottling, chlorosis, mosaic patterns, stunted growth, necrosis and ultimately death of the plant (Ephytia Inrea, 2020).

For conducting the study, we evaluated the phenotypic effect of the treatments in regard of elucidating particularly the biocontrol effect of ePGPBs in reducing the symptoms caused by the tested viruses. Furthermore, the priming action of ePGPB strains were evaluated on healthy plants by the expression levels of defense-related genes to have more clear understanding on the molecular basis behind the observed phenotype.

2. Materials and Methods

2.1. Plant Material and Microbial Strains

2.1.1. Plant material

Plants of *Nicotiana benthamiana* were used as a model plant for the inoculation of different pathogenic and biocontrol agents. Seeds were sown 3 weeks before the experimental trials under greenhouse conditions (25 °C, 72% RH, 16-8h photoperiod) at the Department of Agricultural and Environmental Sciences – Production, Landscape, Agroenergy, Università degli Studi di Milano, Milan, Italy.

2.1.2. Bacterial strains

Four endophytic bacterial strains *Pantoea agglomerans* strain 255-7 (Bulgari et al., 2012), *Pseudomonas syringae* strain 260-02 (Passera et al., 2019), *Paraburkholderia fungorum* strain R8 (Bulgari *et al.,* 2011), *Paenibacillus pasadenensis* strain R16 (Bulgari *et al.,* 2011, Passera et al., 2017) and *Lysinibacillus fusiformis* strain S4C11 were identified, characterized and tested for their biological control efficacy on model plants and host plants.

All the strains were grown either in Lysogeny broth (LB) or on Lysogeny broth Agar (LBA) plates, composed of tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 10 g/L, and agar 15 g/L for the solid medium (Luria and Burrows, 1957), at 24 °C for short-term utilization and were maintained in LB: glycerol (7:3) solution at -80 °C for long-term conservation.

2.1.3. Phytopathogenic Viral strains

Two plant pathogenic virus strains, bought from the Deutsche Sammlung von Mikroorganismen und Sellkulturen GmbH (DSMZ, Germany), were used to evaluate the biological control efficacy of the bacterial strains: *Cymbidium Ring Spot Virus* PV-0272 (CymRSV), *Cucumber Mosaic Virus* PV-0504 (CMV), *Potato Virus X* PV-0017 (PVX) and *Potato Virus Y* PV-1036 (PVY).

The plant pathogenic viral strains were propagated onto 3 weeks old *N. benthamiana* seedlings (10 biological replicates per virus) by following DSMZ virus inoculation protocol. Briefly, the freeze-dried leaves containing the viruses were ground in a mortar with 0.05 M Phosphate-Norit-buffer (pH 7, containing DIECA at 5 mM and EDTA at 1 mM) at a final

concentration of 1:10 w/v and inoculated mechanically onto the top 3 fully developed leaves of *N. benthamiana* seedlings after the application of abrasive carborundum spray. After two weeks of symptoms evaluation, CymRSV (necrotic spots, yellowing and crinkling of leaves, stunted growth) and CMV (irregular mosaic patterns, necrotic areas, crinkling of leaves and stunted growth), PVX (interveinal chlorosis, leaf crinkling, mosaic patterns, necrosis and stunted growth), and PVY (mosaic patterns or mottling, crinkling, and stunted growth), symptomatic leaves were harvested and stored at -80 °C to be used as inoculum source for the viruses in the following assays.

2.1.4. Chitosan-based Resistance inductors

Three different formulation of chitosan-based products; (i) ChitoPlant Solution (CHI-S) produced by Agritalia company, Mantua, Italy, (ii) Chitosan powder (CHT-P) provided by Prof. Alexandr Denisov from the Agrarian University of Novosibirsk, Russia, and (iii) the wettable powder Chitosan hydrochloride (New CHT-S) obtained from Agrilaete company, Palmanova, Udine, Italy were used as a positive reference of resistance induction.

2.2. Biocontrol and Defense related gene expression bioassays

2.2.1. Experimental setup

A total of 5 treatment groups containing different biological replicates were employed in two sets of bioassays, biocontrol and defense related gene expression assay. It comprises of (1) non-treated control plants (NT-C); (2) plants infected mechanically with viral inoculum only; (3) plants treated with bacterial inoculum and then infected mechanically with viral inoculum; (4) plants treated with CHI-S, CHT-P and New CHT-S inductors only (CHI-C and CHT-C, New CHT-C) and (5) plants treated with CHI-S and CHT-P and then inoculated with CymRSV/ CMV (CHI, CHT and New CHT). The number of plants as biological replicates utilized by each treatment in regard of each bioassay are listed in the table 1. This setup was employed for each virus assessed in the study.

Table 3. List of all the biological replicates within the different treatment groups for biocontrol and defense related gene expression assays

Biological replicates of *N. benthamiana*		
Treatments	**Biocontrol assay (n#)**	**Defense related gene expression assay (n#)**
NT-C	10	5
CymRSV/ CMV	10	-
R8, R16, 255-7, 260-02	10	5
CHI-C, CHT-C, New CHT-C	5	5
CHI, CHT, New CHT	5	-

2.2.2. Inoculation of plants with bacterial strains

Liquid suspension of bacterial inoculum was prepared as described in Passera et al., 2019. Briefly, for each strain, 3mL of LB was inoculated with a single, actively growing colony and incubated overnight at 24°C on an orbital shaker at 230 rpm. The next morning, 1mL of the culture media was transferred to a sterile 500mL conical flask containing 100mL of LB broth and left for 8 hours incubation period in the same conditions. The bacterial cells were then pelleted by centrifugation at 4000 rpm for 10 minutes, resuspended in sterile Ringer's solution (Sigma-Aldrich) and diluted to the desired concentration of 10^6 CFU/ml. The inoculum was given as soil drench, pouring 20mL of bacterial inoculum into 7 cm in diameter pots containing potting soil and 3 weeks old *N. benthamiana* seedling. The other plant pots tagged as NT-C, CHI-C, CHT-C, New CHT-C, CymRSV or CMV, CHI, CHT and New CHT were inoculated only with 20mL sterile Ringer's solution.

After 6 days from bacterial inoculation, an aqueous solution of chitosan-based resistance inductors was prepared and 1mL was dispensed by foliar spraying onto the pots containing *N. benthamiana* seedlings (1 plant per pot). The chitosan solutions were prepared following the instructions of the manufacturer: 3mL of CHI-S was added to 147mL of distilled water at a final concentration of 1:50 v/v, whereas, 0.15g of CHT-P was added to 150ml of distilled water to make a homogenized solution (1: 1000 w/v final concentration). And, the New CHT-S was prepared with the concentration of 1:25 w/v by adding 5g of the product into 200 mL at 50°C hot water through the help of a strainer, after a hand mixing with a whisker it was left for 30-40minutes to create high density solvent, then a homogenous solution was made by adding 4mL of the solvent into 96mL of water to reach 100mL of total volume followed by orbital shaking for 5 minutes.

One day after the drenched inoculation of the bacterial strains or the foliar sprayed treatment with chitosan-based products (CHI-C, CHT-C and New CHT-C), leaf samples (0.5 g) were collected from five of the inoculated plants for each strain and the non-treated control (NT-C), to be used for RNA extraction and quantification of gene expression (Section 2.3.2).

2.2.3. Virus inoculation, phenotypic analysis and leaf sampling

The viral strains were mechanically inoculated, as previously described, on the *N. benthamiana* plants (10 biological replicates of treated/ non-treated plants with each bacterial strains and 5 replicates of treated/ non-treated plants with each chitosan inductors) on the day (T0) which corresponds to one week after bacterial inoculation (Vitti et al., 2016; Rendina et al., 2019) and one day after treatment with chitosan (Rendina et al., 2019). After inoculation, the plants were monitored for the following parameters: plant height, number of systemically infected leaves, and symptom severity. The plant height was measured from the surface of the soil to the top node in the plant's stem, not taking into account any foliar laminae or flower buds that reached greater heights. The rate of systemically infected leaves was measured as percentage of symptomatic leaves developed above the virus inoculation site on the total number of leaves present above the virus inoculation site. The symptoms severity was measured by attributing each plant to a symptom severity class from 0 to 5 (where, 0= no symptom; 1= mild leaf curling; 2= moderate leaf curling + mild yellowing and necrotic spots/ mosaic pattern; 3= strong leaf curling + moderate yellowing and necrotic spots/ mosaic pattern + mild stunted growth; 4= strong leaf curling, yellowing, necrotic spots/ mosaic pattern and stunted growth, 5= death of the plant) (Figure 1). The symptom classes were later converted to an infection percentage index (I%) using the formula presented by Townsend and Heuberger (1943). The plants infected with CymRSV were inspected at three different times: T1, 5 days post infection (dpi); T2, 7 dpi; T3, 12 dpi. Whereas, the plants infected with CMV, PVX and PVY were inspected only at T1 and T2. Plant heights were also recorded at the day of inoculation (T0).

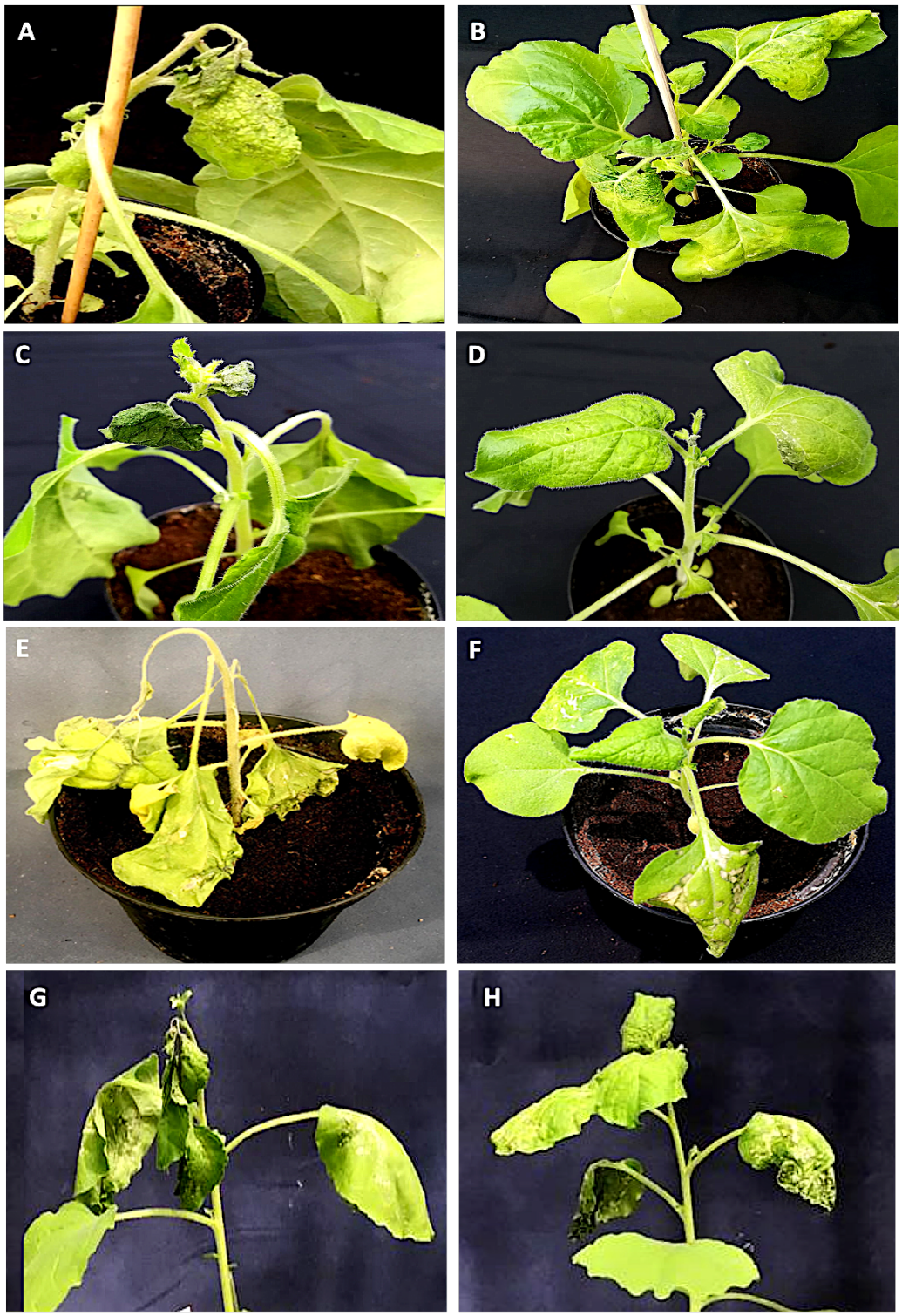

Figure 1. Photographs displaying the symptoms on N. benthamiana plants infected with CymRSV at T3= 12dpi and CMV, PVX and PVY at T2= 7dpi. *A) Non-treated plant infected with CymRSV displaying severe symptoms of class 4 i.e. 72%.* ***B)*** *Plant treated with the strain 255-7 showing milder symptoms of CymRSV infection of class 2 i.e. 38%.* ***C)*** *Non-treated plant infected with CMV displaying severe symptoms of class 4 i.e. 70%.* ***D)*** *Plant treated with the strain R16 showing milder symptoms of CMV infection of class 3 i.e. 52%.* ***E)*** *Non-treated plant infected with PVX displaying severe symptoms of class 4 i.e. 68%.* ***F)*** *Plant treated with the strain 260-02 showing milder symptoms of PVX infection of class 3 i.e. 54%.* ***G)*** *Non-treated plant infected with PVY displaying severe symptoms of class 4 i.e. 78%.* ***H)*** *Plant treated with the strain R8 showing insignificant milder symptoms of PVY infection of class 4 i.e. 79.5%.*

2.3. Relative quantification of RNA

From all the collected samples, both from plants sampled one day after bacterial/chitosan treatment (section 2.2.2) and those infected with the virus (section 2.2.3), total RNA was extracted using the 2% CTAB method described by Gambino *et al*. (2008). This RNA was employed for the relative quantification of selected, relevant transcript. Regardless of the target, the same workflow was used: starting from 1 µg of RNA, real-time PCR assays were performed using a two-steps SYBR® Green approach in a StepOnePlus™ thermocycler (Applied Biosystems™). cDNA synthesis was carried out by reverse transcription PCR in a 20 µL volume reaction using 0.2 µg/ µL random examer primers, 10 mM dNTPs, 0.1 M Dithiothreitol (DTT), 2 µL 10x Retro-transcription Buffer, 1 µL M-MuLV (Moloney murine leukemia virus) reverse transcriptase enzyme and water to reach the required volume. The reaction was carried out with the following thermal cycle: 25 °C for 10 min, 37 °C for 60 min, 70 °C for 5 min, and then kept at 4°C. For qRT-PCR, each reaction was carried out in a 20 µL volume, containing: 2 µL aliquot of cDNA, 400 µM of each primer, 1x Power SYBR® Green PCR Master Mix, and water to reach the volume of 20 µL. Each sample was amplified in triplicates to obtain a more precise value for the threshold cycle.

The obtained Ct values from each treatment were normalized by endogenous plant gene PP2A using the $2^{-\Delta\Delta CT}$ methods described by Livak and Schmittgenin (2001). The Log10 transformation was done on $2^{-\Delta\Delta CT}$ for the optimization of the data scale.

2.3.1. Relative quantification of virus

The viral strains utilized in this study were quantified using specific primer pairs, found in Table 2. For CMV, PVX and PVY, the primers used the sequences reported by Feng and colleagues (2006), Park and colleagues (2009), and Yang and colleagues (2013), respectively. Whereas, the primers for CymRSV were designed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/), targeting the RNA polymerase gene. For the $2^{-\Delta\Delta CT}$ method, a non-treated, virus-infected sample was selected as the reference for the calibration of the data.

Table 4. List of the primers used for the quantification of viruses performed by qPCR assays.

Primers for the Quantification of virus			
Primer Pair	**Sequence 5 '- 3'**	**Fragment (target gene)**	**Bibliography**
PP2A - forward PP2A - reverse	GACCCTGATGTTGATGTTCGCT GAGGGATTTGAAGAGAGATTTC	123bp	Liu et al., 2012
CymRSV - forward CymRSV - reverse	GTA CAT GCG TCA CTT GGG GA TCT CAG CAT CTT CCA ACC GC	195bp (RNA polymerase)	Gennari, 2015-2016
CMV - forward CMV - reverse	CTG GCG ACA ATC TGG AGG GA CGA TAA CGA CAG CAA AAC AC	153bp (Movement protein)	Feng et al., 2006
PVX - forward PVX - reverse	GCC CAA TTG TTA CAC ACC CTA GCC TCA TCT TAA TG	101bp (Coat protein)	Park et al., 2009
PVY - forward PVY - reverse	GGT AGC ACA ACT ATA CGG TGC GAT GTT TGG GGT CGA TCC A	100bp (Coat protein)	Yang et al., 2013

The first column reports the name of the primer, the second sequence of each primer, third the fragment length with targeting protein, and the fourth the reference from which the primer was obtained.

2.3.2. Relative quantification of defense-related genes

Real-time PCR assays were carried out to evaluate the expression levels of three selected defense genes (*Enhanced Disease Susceptibility-1* referred to as EDS1, *Non-expressor of Pathogenesis-related genes-1* referred to as NPR1, and *Pathogenesis-related protein-2B* referred to as PR2B). All the primers utilized in this assay are reported in **Table 3**. For the $2^{-\Delta\Delta CT}$ method, a non-treated sample was selected as the reference for the calibration of the data.

Table 5. List of all the primers used for the relative quantification of gene expression analysis performed by qPCR assays.

Primers for the Gene Expression			
Primer Pair	**Sequence 5 '- 3'**	**Fragment**	**Bibliography**
PP2A - forward PP2A - reverse	GACCCTGATGTTGATGTTCGCT GAGGGATTTGAAGAGAGATTTC	123bp	Liu et al., 2012
NPR1 - forward NPR1 - reverse	GGC CTT GCC TCA TGA TAT TG GCT ACA GCA TAA TGG AGA GC	187bp	Zhang et al., 2012
PR2B - forward PR2B - reverse	CTAAAGAGGGTAGCCCAAGA GTCCCAAACTCCACCAGAGA	147bp	Zhang et al., 2012
EDS1 - forward EDS1 - reverse	GGACAATGGGAGAAGCAGAA GAACGCATCATAATACCCGA	118bp	Zhang et al., 2012

The first column reports the name of the primer, the second sequence of each primer, third the fragment length and the fourth the reference from which the primer was obtained.

2.4. Statistical data analysis

All the data collected from abovementioned bioassays were subjected to statistical analysis by using R-studio, version 3.6.1 (2019-07-05). In particular, the data obtained from the plant heights (described in section 2.2) were analysed with Kruskal-Wallis Anova test followed by P-adjusted values using Benjamini and Hochberg (BH) methods. Whereas, the data obtained from the percentage symptomatic leaves and percentage symptom severity (described in section 2.2) were analysed with non-parametric Wilcoxon rank sum test followed by P- values using Bonferroni methods. The relative quantification data of CymRSV and CMV (section 2.3.1) was analyzed by the parametric T-test with P-adjusted values using Benjamini and Hochberg (BH) methods. Whereas, the data of relative quantification of PVY (section 2.3.1) and defense-related genes (section 2.3.2) was compared with the reference treatment followed by the by non-parametric Wilcoxon rank sum test through P-values using Bonferroni methods. Furthermore, to determine the correlation effect of symptom severity on virus's quantification, a simple linear regression analysis was performed using Linear model function. For all the above-mentioned data analyses, parametric/ non-parametric tests were employed based on the degree of normality of the data checked by Shapiro-Wilk's normality test followed by Levene's test for the homogeneity of variances among treatments.

3. Results

3.1. Effect of ePGPB strains against target viruses

The evaluation of *in-planta* bioassay based on the ability of the ePGPB strains to reduce the incidence of virus infection and to enhance the plant growth (with or without the presence of virus infection) had variable effects, depending on both on the bacterial strains and the viruses. Phenotypic evaluation was performed based on the measurements taken during the experiment: plant heights, percentage of symptomatic leaves, and symptoms severity at different time points. Molecular quantification of virus was based on the readings taken from Log10 $2^{-\Delta\Delta CT}$ values of virus relative quantification at T3 for CymRSV and at T2 for CMV and PVY. However, the quantification of PVX was not possible as all the plants died at the time of sample collection. All of the above-mentioned comparisons were made in between virus infected plants with/ without all bacterial strains, CHI and CHT treatments. The NT-C, CHI-C, CHT-C and New CHT-C treatments were only included in the plant height parameter, as they were not infected with the viruses and showed no symptoms.

3.1.1. Plant heights; a growth promotion parameter

The measurement of plant heights showed no significant differences at T0, regardless of the considered combination of treatment and virus (Figure 2).

Regarding plants inoculated with CymRSV at T1, a slight but statistically significant reduction in height was registered for chitosan treated control plants (CHI-C) and for CymRSV-infected plants treated with CHI and CHT (Figure 2A), in comparison with other treatments and especially the non-treated control (NT-C). At T2, the differences in heights between treatments become greater, leading to the final results visible at T3: in this last observation all bacterial strains gave an increase to plant height compared to the non-treated CymRSV-infected plants restoring height comparable to that of non-infected plants. Whereas, the chitosan treated plants produced similar heights in both non-infected and CymRSV-infected plants (Figure 2A).

For CMV, a similar trend is registered: at T1 the difference in heights is less pronounced, but already shows a trend that is mostly confirmed at T2 (Figure 2B). For CMV, the tallest plants are those that were not infected with CMV (NT-C, CHI-C, CHT-C), while the lowest height is registered for the non-treated CMV-infected plants. With the exception of

strains S4C11 and R8, all other treatments, both bacterial and chitosan-based, allowed the plants to achieve a greater height, even if not comparable to that of healthy plants (Figure 2B).

For PVX, there was no difference observed at T1, but a quite visible difference was observed at T2 (Figure 2C). The tallest heights were observed in both non-infected plants i.e. NT-C, CHI-C and CHT-C, whereas, the lowest heights were observed in the treatments of PVY infected plants, S4C11 and R8 inoculated PVY infected plants. While, all other treatments such as R16, 255-7, 260-02, CHI and CHT showed significantly greater heights (Figure 2C).

Similar trend of PVX was observed in the plants inoculated with PVY at T1 (Figure 2D). However, at T2, none of the treatments from both bacterial and chitosan's showed any significantly different results in the presence of PVY infection. Nevertheless, significantly different and tallest heights were observed only in non-infected plants with either chitosan treated (CHI-C, CHT-C and New CHT-C) or non-treated (NT-C) plants (Figure 2D).

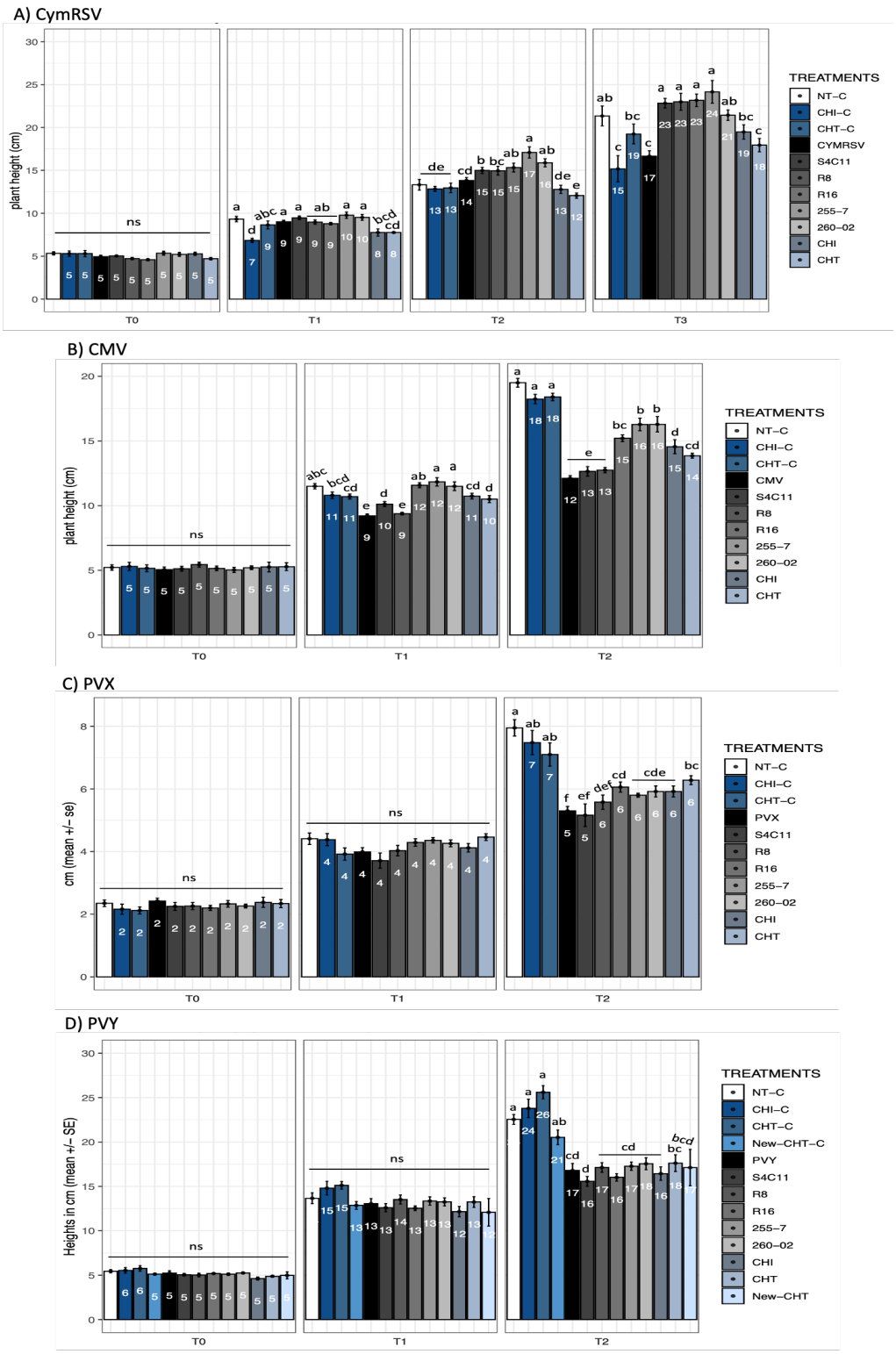

Figure 2. Bar graphs reporting the results of mean of N. benthamiana plants height in cm. Each bar represents a different treatment: *Non-treated healthy control (NT-C), chitosan healthy reference control (CHI-C and CHT-C, and New CHT-C), non-treated virus infected plants (CymRSV, CMV, PVX and PVY), or virus-infected plants treated with either strain S4C11, R8, R16, 255-7, 260-02 or a chitosan-based product (CHI, CHT and New CHT).* ***A)*** *CymRSV-infected plants at different times (T0= Virus inoculation, T1= 5dpi, T2= 7dpi and T3= 12dpi).* ***B)*** *Plants infected with CMV, PVX and PVY at different times (T0= Virus inoculation, T1= 5dpi and T2= 7dpi). Error bars indicate standard error. Different letters (a-f) indicate statistically significant differences ($p < 0.05$) in the results to Kruskal-Wallis non-parametric test followed by P-adjusted values using Benjamini and Hochberg (BH) methods.*

3.1.2. Systemic symptomatic leaves and symptom severity; biocontrol parameters

The percentage of systemic infection of leaves was evaluated as a parameter that describes the ability of the virus to move inside the infected host plant. The statistical analysis was conducted with the non-parametric Wilcoxon rank sum test followed by P-adjusted values based on Bonferroni methods using the target viruses as reference of comparison. Based on the overall obtained results, it was observed that all of the viruses (CymRSV, CMV, PVX and PVY) spread to most of the leaves of the plants (systemic infected leaves percentage >90%) when no treatment was applied (Figure 3).

For CymRSV, there was no significant differences between the percentage of systemic infected leaves in different treatments at T1 despite the great difference in the actual values (ranging from 67 to 92%). At the following timepoints, the number of systemic infected leaves consistently rose in the non-treated CymRSV-infected plants, up to 100% at T3 (Figure 3A). In contrast, the percentage decreased notably in all treated plants, becoming around 40% for treatments with R8, R16 and CHI, indicating that the virus did not spread to the newer leaves that were produced.

The percentage of systemic infected leaves of CMV showed no statistically significant difference between treatments, with the exception of strains R16 and 260-02 at T1. Moreover, the strain 260-2 was consistently able to give lower percentage of infected leaves even at T2 exhibiting the reduction of around 30% as compared to non-treated CMV infected plants (Figure 3B).

For PVX, the percentage of systemic infected leaves showed no statistically significant difference between treatments, except for strain R16, which gave lower percentage of infected leaves both at T1 (non-significantly) and T2 (significantly) (Figure 3C).

The systemic infection reached higher in the plants infected with PVY including the non-treated, bacterial treated and CHI treated plants. Only the plants treated with CHT and New CHT gave reduction in systemic infection to newly produced leaves as compared to non-treated PVY infected plants (Figure 3D).

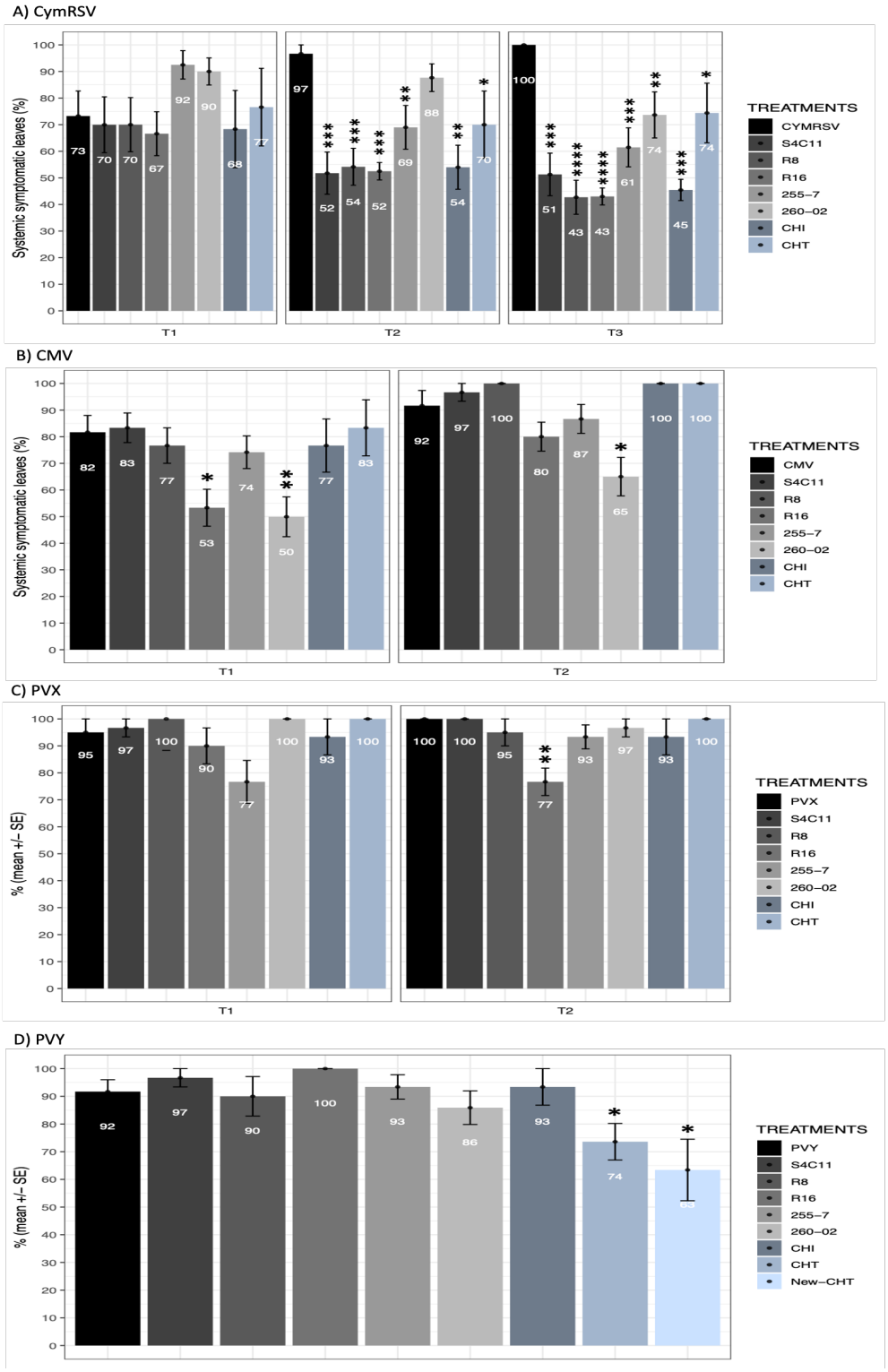

Figure 3. Bar graphs reporting the results of mean percentage values of systemic symptomatic N. benthamiana leaves. Each bar represents a different treatment: *Non-treated virus infected plants (CymRSV, CMV, PVX, PVY), and virus-infected plants treated with either strain S4C11, R8, R16, 255-7, 260-02 or a chitosan-based product (CHI, CHT, New CHT).* ***A)*** *CymRSV-infected plants at different times (T1= 5dpi, T2= 7dpi and T3= 12dpi).* ***B)*** *CMV-infected plants;* ***C)*** *PVX-infected plants; and* ***D)*** *PVY-infected plants at different times (T1= 5dpi and T2= 7dpi), respectively. Error bars indicate standard error. Asterisks indicate significant difference among results according to the non-parametric Wilcoxon rank sum test followed by Bonferroni methods compared with virus-infected non-treated plants (* for p < 0.05, ** for p <0.01, *** for p <0.001, **** for p =0.000).*

The severity of symptoms was evaluated to describe the ability of the inoculated virus to cause symptoms when the plants underwent different treatments. Statistical comparison was made between the different treatments in reference to the prospective virus using the non-parametric Wilcoxon rank sum test followed by P-adjusted values based on Bonferroni methods after checking the normal distribution of data and homogeneity of variances among treatments.

The plants infected with CymRSV showed low severity of symptoms at T1, with statistically significant differences in the treatments R16, 255-7 and 260-02 (I%I: 6-12%) as compared to other treatments with no significant difference (I%I: 16-26%) (Figure 4A). At T2, the severity of symptoms in non-treated CymRSV-infected plants changed only slightly from the previous measurement, while the symptom severity greatly increased in all other treatments especially in 260-02 and CHT treated CymRSV-infected plants. At T3, symptoms were fully developed on non-treated CymRSV-infected plants with a symptom severity of 72%; all other treatments, with the exception of CHT, managed to exert a statistically significant reduction of symptom severity, as low 22% for the treatment with strain R16 (Figure 4A).

For CMV, the evaluation of symptoms at T1 showed a lower severity for non-treated CMV-infected plants, with statistically significantly higher severity percentages for plants treated with R16 strain, CHI, and CHT (Figure 4B). At the second time point, the symptoms on the non-treated CMV-infected plants were the highest (70%), and the plants treated with strains S4C11, R16, 255-7, and 260-02 showed statistically significantly lower symptom severity (Figure 4B).

The assessment of PVX infected plants indicated non-significant symptoms severity among all the treatments, regardless of bacterial and chitosan inoculation at all time points. With an exception of strain 260-02, that was able to significantly reduce the severity of symptoms of up to 54% at T2 while the non-treated PVY-infected plants showing percentage symptoms severity up to 68% (Figure 4C).

On the contrary, non-treated PVY-infected plants exhibited symptoms that were recorded highest at T1, whereas, the strains R8, R16 and 260-2 showed statistically significantly lower symptom severity as shown in the figure 4D. Then, at T2, plants treated with the strains S4C11, R16 and 260-2 rose significantly as compared to non-treated PVY-infected plants, with an exception of the plants treated with CHT and New-CHT, that were shown to lower the severity of symptoms (Figure 4D).

In accordance with these results, non-treated CymRSV-infected plants displayed severe symptoms, causing crinkling, yellowing and necrotic spots on inoculated leaves (Figure 1A).

Whereas plants inoculated with strain 255-7 and infected with CymRSV displayed significant improvement and milder symptoms (Figure 1B). The symptoms of CMV infection were very intense and caused strong leaf crinkling, yellowing, mosaic patterns and death of the systemic leaves (Figure 1C). In contrast, the plants treated with the strain R16 and infected by CMV displayed slight improvement and milder symptoms after the infection (Figure 1D). As for PVX, the symptoms of both treated and non-treated plants were shown adverse symptoms and exhibited necrosis, irregular spotting all over the leaves, stunted growth and caused death of the plants within 10 days post inoculation (Figure 1E). Except for the plants that were treated with strain 260-02 that presented significant milder improvement in symptoms severity as shown in the figure 1F. The symptoms of PVY infection were observed much stronger even in the plants treated with the ePGPB strains, causing overall irregular mosaic patterns, leaf crinkling, stunted growth and death of the growing points of the plant (Figure 1G). Even with the non-significant differences among treated and non-treated PVY infected plants, bacterial strains were showing minor improvements in the newer plants as shown in the figure 1H for strain 255-7 as a comparison with infected plants.

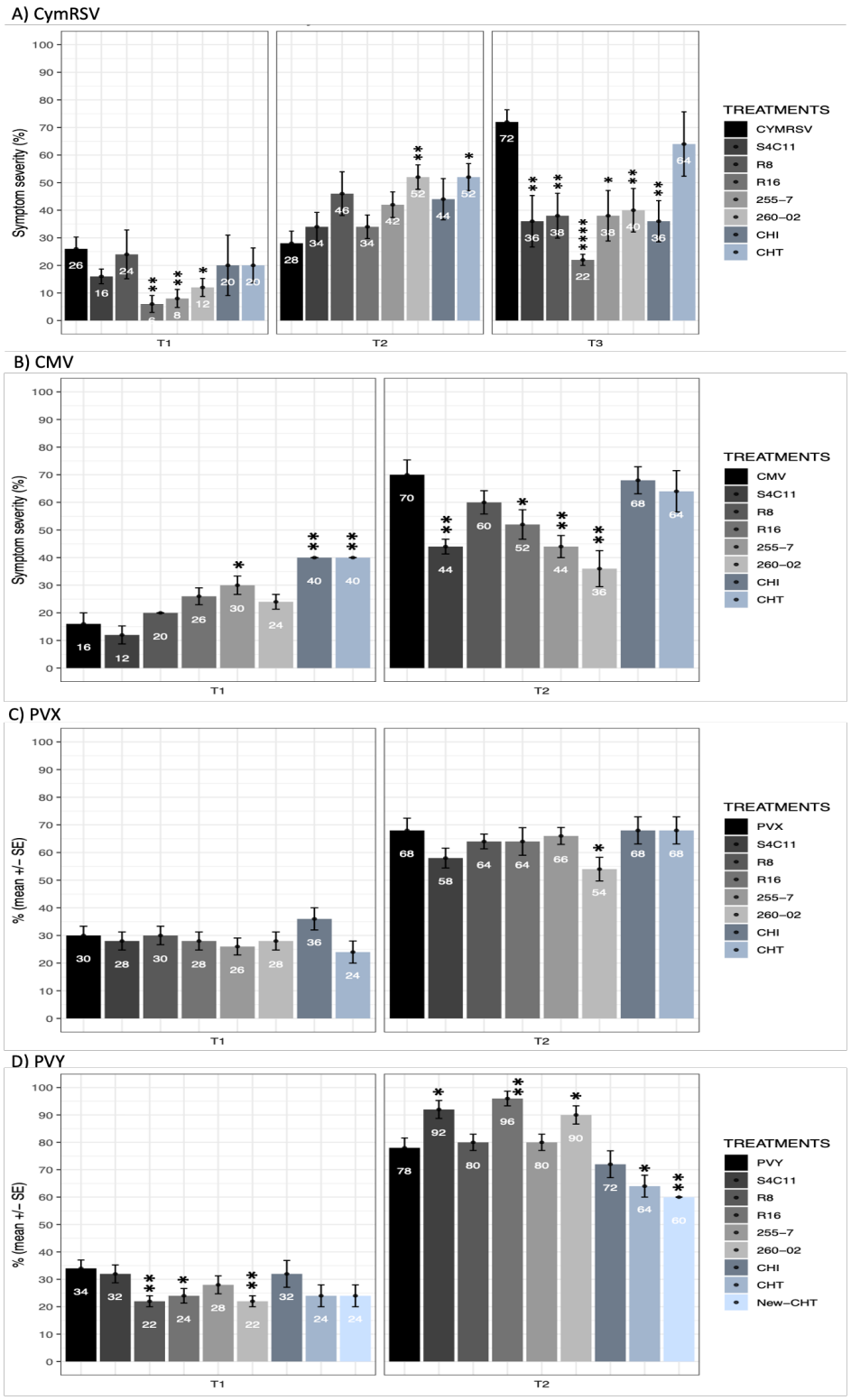

Figure 4. Bar graphs reporting the results based on mean percentage values of symptom severity. Each bar represents a different treatment: *Non-treated virus infected plants (CymRSV, CMV, PVX, PVY), and virus-infected plants treated with either strain S4C11, R8, R16, 255-7, 260-02 or a chitosan-based product (CHI, CHT, New CHT).* ***A)*** *CymRSV-infected plants at different times (T1= 5dpi, T2= 7dpi and T3= 12dpi).* ***B)*** *CMV-infected plants;* ***C)*** *PVX-infected plants; and* ***D)*** *PVY-infected plants at different times (T1= 5dpi and T2= 7dpi), respectively. Error bars indicate standard error. Asterisks indicate significant difference among results according to the non-parametric Wilcoxon rank sum test followed by Bonferroni methods compared with virus-infected non-treated plants (* for p < 0.05, ** for p <0.01, *** for p <0.001, **** for p =0.000).*

3.1.3. Relative quantification of virus

The quantification of the virus was carried out to discriminate whether the treatments had an effect on the ability of the virus to replicate in the host, or on the ability of the virus to cause symptoms, without affecting its concentration.

The results of T-test gathered from the fold change ($Log_{10}2^{-\Delta\Delta CT}$) values of relative quantification of CymRSV at T3, demonstrated equal amount of virus load present among all treatments except for the strain S4C11 ($p < 0.005$), R8 ($p < 0.000$), 255-7 ($p < 0.000$) and CHT ($p < 0.05$) that significantly lowered the concentration of virus within the plant (Figure 5A).

For CMV, the results obtained from T-test indicated that the quantity of virus was significantly lower in the leaves of plants treated with strain 255-7 ($p < 0.05$), CHI ($p < 0.000$) and CHT ($p < 0.05$) as compared to non-treated CMV-infected plants. It is interesting to note that in the case of CMV-infected plants treated with CHI, the concentration of virus was significantly higher than the control plants, while in all other cases the difference in concentration is always given by a decrease in concentration. All other treatments had no statistically significant difference in virus concentration from non-treated plants (Figure 5B).

The results of quantification of PVY as analysed from the Wilcoxon rank sum test was seen with great significant reduction in all the plants treated with both ePGPB strains and chitosan-based products as compared to non-treated PVY-infected plants. The significance level of (P-value < 0.05) was observed in the treatments S4C11 and New CHT, whereas, all other treatments showed significant differences with (P-values < 0.005) as compared to non-treated PVY-infected plants (Figure 5C).

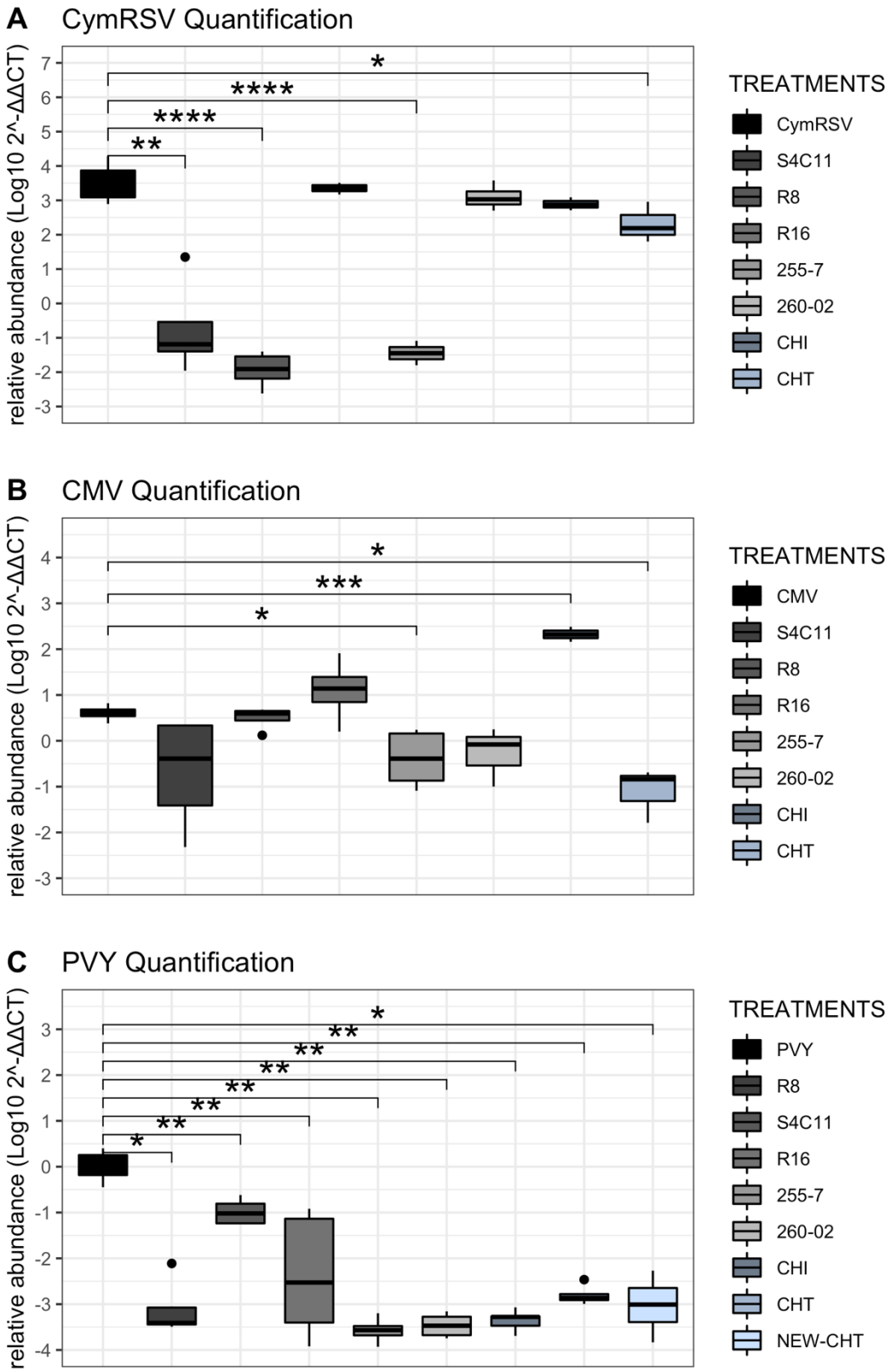

Figure 5. Boxplot indicating fold change ($Log_{10}2^{-\Delta\Delta CT}$) values of A) CymRSV quantification under different treatments sampled at T3. *Both* ***B)*** *CMV quantification; and* ***C)*** *PVY quantification under different treatments sampled at T2. Asterisks indicate significant difference among results according to the t-test for CymRSV and CMV, followed by P-adjusted values using Benjamini and Hochberg (BH) methods; and according to the non-parametric Wilcoxon rank sum test for PVY, followed by Bonferroni methods compared with virus-infected non-treated plants (* for p < 0.05, ** for p <0.01, *** for p <0.001, **** for p =0.000).*

3.2. Effect of symptom severity on virus quantification

In order to understand if there was an effect of symptoms severity on the concentration of virus in the plant, a linear regression analysis was performed using the percentage of symptom severity as an independent variable and $Log_{10}2^{-\Delta\Delta CT}$ values of virus quantification as a dependent variable at T3 for CymRSV, and at T2 for CMV and PVY.

The results obtained from these data indicate that there is no statistically significant correlation between the symptom observed on a plant and the quantity of virus detected in the sample coming from that same plant (Figure 6). This is especially true for CymRSV ($P = 0.23$, $R^2 = 0.021$) and PVY ($P = 0.42$, $R^2 = 0.008$), as samples with the same symptom severity can have either very high or very low abundance of virus (Figure 6A, 6C). In an exception with the CMV samples, that are showing statistically significant correlation between the values ($P = 0.014$, $R^2 = 0.085$), suggesting an increase in concentration as the symptoms become more severe (Figure 6B).

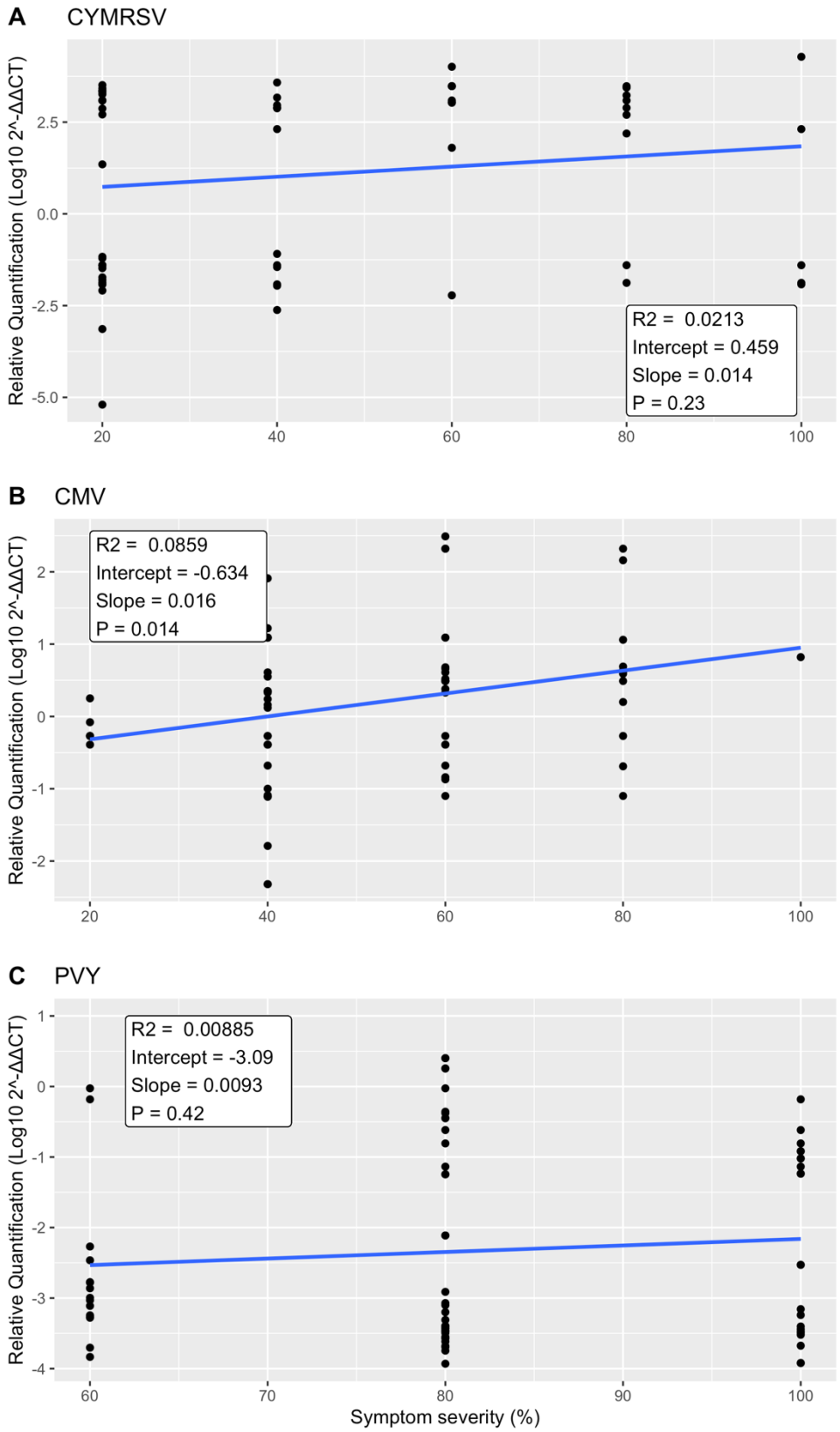

Figure 6. Regression analysis representing the effect of percentage symptoms severity on X-axis and Log10 ratio of virus quantification on Y-axis in each treatment respectively*. **A)** CymRSV infected plants at T3= 12dpi; **B)** CMV-infected plants and **C)** PVY-infected plants at T2= 7dpi. R-squared values presenting the goodness-of-fit measure of the model and P-values showing the significant difference ($p < 0.05$) between each treatment.*

3.3. Effect of bacterial strains on defense-related genes

The evaluation of gene expression was conducted on plants treated with ePGPB strains, chitosan-based products or non-treated as a control, respectively, at 24 hours post inoculation, before infecting with the virus. This analysis allowed the evaluation of the regulation of plant defense-related genes by the treatments, in absence of a pathogen. Statistical comparison was made between the different treatments in reference to NT-C using the transformed $Log_{10}2^{-\Delta\Delta CT}$ values based on three defense genes (NPR1, PR2B and EDS1). Significant differences were determined using a non-parametric Wilcoxon rank sum test followed by Bonferroni test after checking the normal distribution of data and homogeneity of variances among treatments.

The results obtained from the genes NPR1 indicated no significant differences among treatments as compared to non-treated control (Figure 7A). For the gene PR2B, a statistically significant decrease in the abundance of the transcript was seen for plants treated the strain S4C11 ($P < 0.05$), however, the vice-versa was observed with CHT-C treated plants ($P < 0.005$) (Figure 7B). The gene EDS1 underwent upregulation in the plants treated with strain 260-02 ($P < 0.05$), CHI-C ($P < 0.05$), New CHT-C ($P < 0.05$) and increased even more in the plants treated with strain R16 ($P < 0.005$) (Figure 7C).

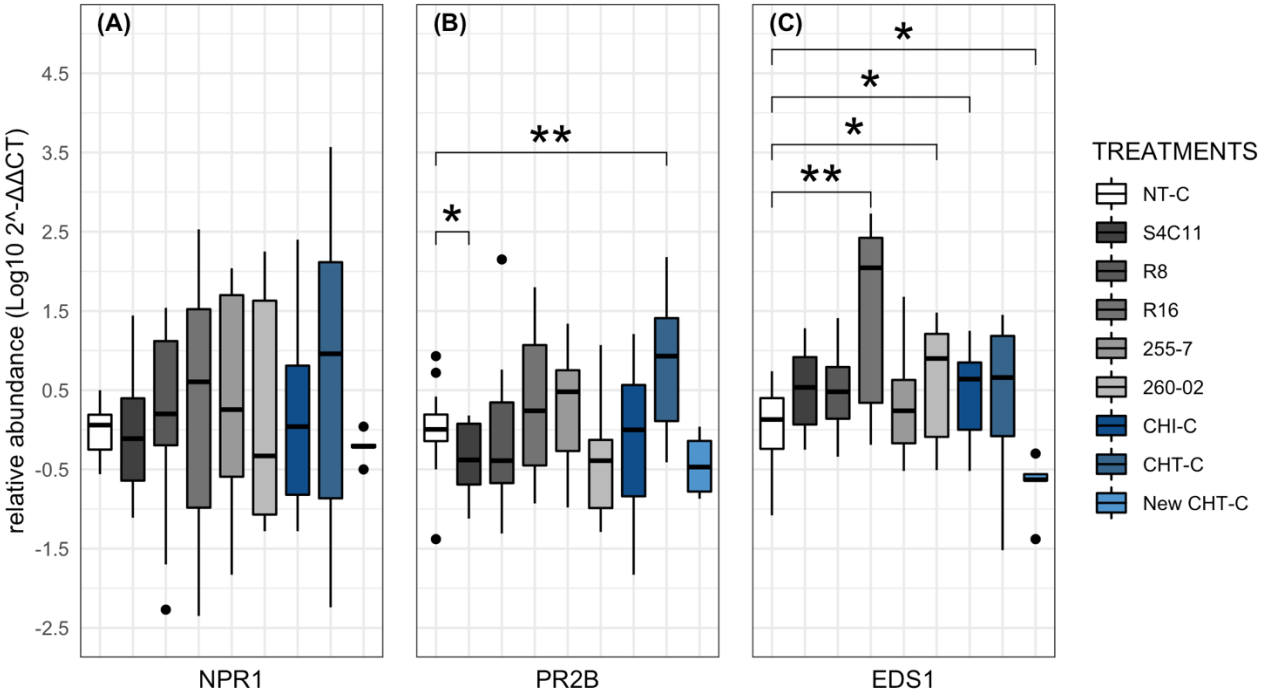

Figure 7. Relative gene expression assay in N. benthamiana plants (treated and non-treated) with ePGPB strains along with chitosan indicators (CHT-C, CHI-C, New CHT-C) sampled at 24 hpi. ***A)*** *NPR1,* ***B)*** *PR2B, and* ***C)*** *EDS1. Y-axis represents* $2^{-\Delta\Delta CT}$ *value expressed as log10 of each gene, normalized on PP2A gene, and X axis represents different treatments. Bars representing standard error of mean values at 95% confidence interval. Asterisks indicating significant difference according to non-parametric Wilcoxon rank-sum test followed by P values based on Bonferroni methods compared with non-treated group (* for* $p < 0.05$*, and ** for* $p < 0.01$*).*

4. Discussion

Endophytic plant growth promoting bacteria (ePGPB) have been utilized for years as a mean to promote plant growth and reduce the damage caused by bacterial and fungal pathogens with a lower environmental impact than synthetic products, but their potential against viral diseases has not been explored as much until recently (Le Cocq et al., 2018). The present study was aimed to investigate the possible biocontrol effect of selected ePGPB strains, some of which have already been characterized for their great potential of enhancing plant growth and ameliorating various biotic stresses (Passera et al., 2017; Passera et al., 2019; Passera et al., 2020), against the viruses *Cymbidum ringspot virus* (CymRSV) and *Cucumber mosaic virus* (CMV) in *N. benthamiana* plants. In addition to the bioassay, treatments with chitosan-based products were used as a positive reference of resistance inductors to compare their effect, based on phenotypic and on gene expression of ISR defense pathway, to that of the bacterial strains.

In the present study, we found that the strains S4C11, R8, R16, 255-7 and 260-02 displayed plant-growth promotion ability towards *N. benthamiana*. For strain 260-02, this is in accordance with previous results that demonstrated its ability to promote growth in bell pepper and tomato, other plants of the Solanaceae family (Passera et al., 2019).

While all five strains allowed CymRSV-infected plants to grow to a height comparable or even taller than non-infected controls, only three strains (R16, 255-7, 260-02) promoted growth in CMV-infected and PVX-infected plants. These findings, with the exception of strains S4C11 and R8, were in agreement with Kumar et al (2016), who reported growth promotion of CMV-infected tobacco plants with the application of *Paenibacillus lentimorbus* B-30488 strain in the soil. It is notably promising that these strains depict the growth promoting capabilities even under various biotic stress. Though, the plant growth promotion of the strains was lesser in plants infected with CMV and PVX, suggesting that the symptoms induced by CMV and PVX, but not CymRSV, may have interference with the strains' ability to promote plant growth. The similar reason could be possible in case of plants infected with PVY, where none of the strains contribute to plant growth promotion.

On the other hand, the effectivity of chitosan treatments to promote growth is confined to CMV and PVX, and they were not effective in CymRSV-infected and PVY-infected plants. The results of chitosan against CMV and PVX were related with the studies of Abd El-Gawad and Bondok (2015), who reported the improvement in vegetative growth of tomato plants against *Tomato mosaic virus* with the application of chitosan. Similar findings were reported

by Firmansyah et al (2017), indicating positive effect of chitosan on growth promotion in cucumber plants infected with squash mosaic virus.

The parameters evaluated to describe the progress and severity of viral infection in the host plants were (i) percentage of leaves systemically infected, indicating the spread of the virus inside the host plant; (ii) the symptom severity, indicating the ability of the virus to produce symptoms in the host plant; and (iii) the virus quantity, indicating the ability of the virus to reproduce in the host plant. The first two, phenotypic parameters, are in accordance with all treatments and viruses: while the values of the two indexes may not be similar or follow a specific trend, a statistically significant reduction, in comparison to the non-treated control, in one parameter is always accompanied by a statistically significant reduction also in the other. This result suggests that the overall amelioration of the host plant health contributes both to counteract the expression of symptoms and the diffusion of the virus inside the host. The third parameter, virus quantification, instead does not show a clear correlation to the phenotypic ones: in some cases, (i) the reduction of symptom severity is accompanied by a decrease in virus concentration (such as: CymRSV-infected plants treated with strains S4C11, R8 or 255-7; CMV-infected plants treated with strain 255-7; and PVY-infected plants treated with CHT and New CHT); (ii) the reduction of virus concentration is not accompanied by a decrease in symptom severity (such as: CymRSV-infected plants treated with CHT; CMV-infected plants treated with both CHI and CHT; PVY-infected plants treated with all ePGPB strains); (iii) or the opposite, in which the concentration of the virus is not reduced, but the symptom severity is greatly reduced (such as: CymRSV-infected plants treated with strain R16 and 260-02; CMV-infected plants treated with strains S4C11 and 260-02). The most unexpected result recorded was that there are no differences in symptoms compared to the non-treated control, but either the concentration of virus is much higher (CMV-infected plants treated with CHI) or the concentration of virus is much lower (PVY-infected plants treated with bacterial strains).

The results obtained regarding strain 260-02 are in accordance and add to those described by Passera et al (2019), in which the biocontrol effect of strain 260-02 in pepper plants mechanically inoculated with CymRSV was reported. The present study extends these results to a new host (*N. benthamiana*) and new pathogens (CMV and PVX).

The treatments with the commercial chitosan-based product CHI proved to be effective against CymRSV and PVY but not against CMV and PVX infected plants. The results of PVY were in accordance but the results of PVX were found to be in contradiction with the studies

conducted by Chirkov and colleagues (2001), as they reported that the spraying with chitosan provided resistance in potato plants against virus's infection. Moreover, these results are partially in accordance with those presented by Kumar and his co-workers (2016) which report that chitosan resistance inductors led to a significantly higher plant height in the CMV infected plants, but not to a significant reduction in symptoms. To the best of our knowledge, the effect of chitosan treatments against CymRSV is reported for the first time in this study.

In this study, all of the viral pathogens started to manifest symptoms at 5 days after inoculation in all plants, both treated and non-treated. Several previous works, carried out both using bacterial biocontrol agents and chitosan-based products, reported that part of the biocontrol effects was a delay in symptom manifestation, that could range from 11 to 20 days (Raupach et al., 1996; Kumar et al., 2016; Rendina et al., 2019). The difference between the results of this study and the above-mentioned results could be due to of the use of different bacterial strains, chitosan formulations, viral strains or even different plant species. In particular, *N. benthamiana* has been utilized as a model host plant for viral infection especially for its impressive susceptibility against the variety of plant viruses, compared to that of natural host plants (Liu et al., 2012; Nakasugi et al., 2013; Bally et al., 2015).

The effect and relevance of virus concentration on the symptoms caused is still a matter of debate in the scientific community, with published studies showing both that there is correlation between the two parameters (Guo et al., 2019), and that no correlation is present (Raupach et al., 1996; Thakur, 2017). The present study reinforces the idea that no strict correlation is present between virus concentration and symptom severity, but also this result could be dependent on the particular combination of biocontrol agents, viral strains, and plant host utilized.

The last objective of the present study was to understand a possible mechanism of action behind the biocontrol effect that was registered towards virus. Considering the unique nature of viruses, the most likely biocontrol trait involved is the induction of the host plant's defenses. The elicitation of defense-related genes mediated by ePGPBs have been studied in numerous studies to understand which signaling pathways are associated with the induction of systemic plant resistance (Conrath et al., 2015; Martinez et al., 2016). The present study analyzed a set of three defense-related genes (Zhang et al., 2012): EDS1, an upstream gene in the salicylic acid (SA) signaling pathway (Wiermer et al., 2015); NPR1, a master regulator gene that mediates the cross-talk between the pathways related to SA or jasmonic acid (JA)

and ethylene (ET) (Spoel et al., 2003); and PR2B, pathogenesis-related protein which is a molecular marker of systemic acquired resistance (SAR) (van Loon et al., 2006).

The results obtained from the plants treated with strains R16 and 260-02, which gave the greatest reduction in symptom severity on CymRSV and CMV respectively, showed an up-regulation of the EDS1 gene, without having a significant impact on NPR1 and PR2B genes, as compared to non-treated control plants. These results suggest that the mechanism involved could be related to SA-dependent ISR pathways, as reported previously for other strains belonging to the genera *Paenibacillus* and *Pseudmonas* (Vlot et al., 2009; Pieterse et al., 2014). Furthermore, Beris and colleagues (2018) demonstrated that the activation of SA-independent defense pathways by cell-free culture filtrate of *Bacillus amyloliquefaciens* strain MBI600 did not trigger NPR1 and EDS1 gene expression activation in tomato plants. This could be explanation for the strain S4C11, that was shown to downregulate the PR2B transcripts without having a triggering impact on NPR1 and EDS1 gene expressions.

Strains R8 and 255-7, in contrast, did not show upregulation for any of the investigated genes. This might suggest that they either affect different pathways in the plant host, or only act as plant growth-promoting agents, providing a benefit to plant health that is dependent on nutrition only; but it must be considered that the application of ePGPBs in uninfected plants does not necessarily induce defense-related gene expression, instead they often triggered with the pathogens and pests attack (Pieterse et al., 2014). Therefore, carrying out the gene expression analyses on plants that received both bacterial and virus inoculum could better unveil the hallmarks of ISR facilitated by ePGPBs, but at the same time add a layer to the interaction, making the results more difficult to analyze and interpret.

Regarding the chitosan-based resistance inducers, CHI-C showed an upregulation in EDS1 gene only, apparently having the same effect as the treatments with strains R16 and 260-02, suggesting the activation of a SA-dependent pathway. In contrast, treatment with CHT-C showed an upregulation of PR2B gene and a slight, and non-statistically significant, increase in the expression of NPR1. These results are in accordance with those reported by Chirkov (2002) as well as Redina and colleagues (2019). Whereas, the results of New CHT-C treatment indicated a contradiction from the findings of Beatrice and colleagues (2017) that indicated the higher expression level of PR1 and PR5 genes by chitosan treatment in Kiwifruit plants, as the present study showed the slight downregulation of the New CHT-C treatment in plant that was not statistically significant among NPR1 and PR2B genes, but it was evidently significant in

EDS1 gene, which suggests an indication of the activation of Ethylene (ET) or Jasmonic acid (JA) dependent pathways.

5. Concluding Remarks

The results obtained in the present study indicate that the selected bacterial strains show promise in being able to reduce the symptoms caused by all four different single-stranded RNA phytopathogenic viruses. Still, future studies will be needed to clear several questions that remain open regarding this biocontrol effect, such as (i) how long is the protection afforded by the treatment with the bacteria, (ii) the epidemiological implications of these treatments, considering that the plants showing milder symptoms can still act as sources of inoculum in field, and (iii) go more in-depth in the study of the gene expression patterns associated to the treatments, both by analyzing more genes, by considering more time-points, and include the plant-bacteria-virus interaction in the experiment to describe the plant's response.

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2

Preliminary study on the induction of Tomato Aspermy Virus (TAV) resistance by exogenous application of coat-protein derived dsRNA molecules

The contents of this chapter require further study for the publication

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Abstract

Phytopathogenic viruses cause severe damage on cultivated plants that results in heavy yield loses and affects global food security, and hence demanding for effective control measures. Emerging crop protection strategies involve the implementation of double-stranded RNAs (dsRNA) that mediates RNA interference to induce post-transcriptional gene silencing (PTGS) in virus challenged plants. In the present study, we used non-transgenic strategy to induce resistance against *Tomato Aspermy Virus* (TAV) in *Nicotiana benthamiana* plants. dsRNA molecules targeting the gene encoding for coat protein (CP) gene was produced by a two-step PCR assay followed by *in vitro* transcription and purification. The inoculum mixture was prepared from the solution buffer containing TAV sap diluted at ratios of 1:5 and 1:10, that was utilized either alone as TAV inoculum or with *in vitro* produced dsRNA molecules derived from CP gene of TAV. Upon exogenous application, none of the dsRNA inoculated treatments showed significant reduction in severity of symptoms at any time point (6, 12, 18 days post inoculation) . However, the dsRNA managed to uphold the plant heights significantly at dilution factor (1:10) until 12 days of post inoculation as compared to TAV infected plants. Further studies are required to fill the gaps of present study and enhance the experimental procedure that may have been involved in lack of efficacy to induce resistance against TAV infection.

Keywords: *Double-stranded RNA (dsRNA), Tomato Aspermy Virus (TAV), Nicotiana benthamiana, Coat protein (CP) gene, Post-transcriptional gene silencing (PTGS), RNA interference (RNAi).*

1. Introduction

Since the dawn of agriculture, plants are facing threats from virus infections that lead to substantial losses in the crop yield and fruit quality, affecting adversely the human well-being and biodiversity conservation (Souiri et al., 2020). Phytopathogenic viruses consist of one or more nucleic acid molecules, either single or double strands of DNA or RNA, protected by the coat proteins. Once they get entry inside the host plant, they replicate in individual cells by modifying and using host's replication machinery and spreads progressively and systemically between the cells and vascular systems (Abdelkhalek and Hafez, 2020). One of the most phytopathogenic type is RNA virus's family *Bromoviridae*, that has members distributed worldwide causing diseases to a host's range of approximately 10,000 plant species. Among this family, a very relevant genus is *Cucumovirus*, characterized by positive single-stranded RNA viruses, and boasting four broad range type member species; *Cucumber mosaic virus* (CMV), *Gayfeather mild mottle virus* (GMMV), *Peanut stunt virus* (PSV), and *Tomato aspermy virus* (TAV) (ICTV Master Species List 2018b.v2., 2019).

In particular, TAV isolates solely infects broad range of plant species belonging to dicots and monocots families such as Solanaceae, Leguminosae-Papilionoideae, Chenopodeaceae, Liliaceae, Cucurbitaceae, Cannaceae, Compositae, and many others. TAV causes wide-ranging symptoms that vary from plant to plant, including mild to severe mosaics patterns, malformation, seedless fruits, dwarfing, and deformation of flowers (Raj et al., 2007). This virus is primarily transmitted by aphid vectors, but can also infect through mechanical inoculation and be vertically transmitted by seeds. (Gupta and Singh, 1981). TAV is a tripartite virus, and the dicistronic RNA3 encodes 3a movement protein (cell-cell movement) and coat protein (encapsidation) that is expressed from a subgenomic RNA, responsible for TAV mobility in host plants, insect transmission, expression of symptom and host range (Kaplan et al., 1998; Palukaitis et al., 1992).

However, in a counter-defense, plants have developed highly complex and multi-layered immune system to combat virus attack by inducing diverse defense mechanisms, such as: innate immunity, RNA silencing, translational repression, non-sense-mediated decay, ubiquitination-mediated protein degradation, non-stop and no-go decays etc. (Carr et al., 2019; Garcia et al., 2014; Gouveia et al., 2017; Moon and Park, 2016; Szádeczky-Kardoss et al., 2018b, 2018a).

Scientists have utilized these mechanisms to induce resistance in plants to combat viruses. Specifically, RNA silencing-based technology have progressed as an incredible

defense mechanism that is triggered by RNA interference (RNAi) mediated by sequence-specific degradation of complementary mRNA transcripts (post-transcriptional gene silencing, PTGS), which is highly conserved among high eukaryotes and playing a crucial role in growth, development, host defence against viruses (Carthew and Sontheimer, 2009). For example double-stranded RNAs (dsRNAs) derived from viral genes have been utilized as nucleic acid pesticide protection in the field (Zotti et al., 2018). The review conducted by Dubrovina and Kiselev (2019) highlighted the major studies that have reported that exogenously applied dsRNAs conferred the induction of resistance to several host species, including tobacco, papaya, tomato, cowpea, watermelon, cucumber, squash, maize against different viruses such as *tobacco mosaic virus* (TMV), *tobacco etch virus* (TEV), *pepper mild mottle virus* (PMMoV), *alfalfa mosaic virus* (AMV), *zucchini yellow mosaic virus* (ZYMV), *papaya ringspot virus* (PRSV), *bean common mosaic virus* (BCMV), and more. Many of above-mentioned studies reported the induction of plant resistance against target viruses by the foliar application of dsRNAs and in some cases by utilizing dsRNAs derived from amplified viral protein sequences (Carbonell et al., 2008; Konakalla et al., 2016; Mitter et al., 2017b; Tenllado and Dıaz-Ruız, 2001; Worrall et al., 2019). As an example, the most recent study conducted by Vadlamudi and colleagues (2020) demonstrated that CP and HC-Pro genes of PRSV-Tirupati isolate derived dsRNA molecules conferred 100% resistance against PRSV-Tirupati infected papaya plants and 94% and 81% resistance against PRSV-Delhi infected papaya plants, respectively.

In the present study, a non-transgenic approach was employed in order to induce resistance against TAV in *Nicotiana benthamiana* plants. More precisely, *in vitro* produced dsRNA molecules derived from the CP genes of TAV were exogenously applied onto *Nicotiana benthamiana* plants, as described previously (Kaldis et al., 2018; Konakalla et al., 2016; Voloudakis et al., 2015). In this context, the investigations involved were the evaluation of phenotypic parameters; plant heights and symptoms severity; to test their efficacy in protecting them against TAV infection.

2. Material and Methods

2.1. Plant material

Three weeks old *Nicotiana benthamiana* seedlings were used as a model host, grown under greenhouse conditions (25 °C, 72% RH, 16-8h photoperiod) at the Department of Agricultural and Environmental Sciences – Production, Landscape, Agroenergy, Università degli Studi di Milano, Milan, Italy.

2.2. TAV propagation

The TAV strain (*Tomato Aspermy Virus* PV-0220) used in this study was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany). The inoculum was further propagated onto 3 weeks old *N. benthamiana* seedlings (10 biological replicates) by following DSMZ virus inoculation protocol. Briefly, the freeze-dried leaves containing TAV were ground in a mortar with 0.05 M Phosphate-Norit-buffer (pH 7), containing 5 mM DIECA, and 1 mM EDTA at a final concentration of 1:10 w/v. The crude mixture was mechanically inoculated onto three fully developed leaves of *N. benthamiana* seedlings (1 mL per plant) after the spray application of abrasive carborundum powder. The symptoms such as mosaic patterns, crinkling, leaf rolling, and stunted growth, was observed for two weeks and the symptomatic leaves were collected and stored at -80 °C to be used as inoculum source for the experiment.

2.3. In vitro dsRNA TAV production

2.3.1. TAV target sequences for dsRNA production

The primer pairs for TAV strain were designed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/), targeting the coat protein (CP) gene. The primers used to amplify this gene fragment are as follows: TAV_F (5'- TCACCACTGTCACACACTCT-3') and TAV_R (5'- CGTTAGCTGGATGGACAACC-3').

2.3.2. Target DNA fragments amplification, T7 promoter sequence incorporation and in vitro transcription

The *in vitro* production of dsRNA molecules derived from CP gene was achieved by a two-step polymerase chain reaction (PCR) procedure, followed by *in vitro* transcription, as

described previously (Kaldis et al., 2018; Konakalla et al., 2016; Voloudakis et al., 2015), with some modifications, in a total volume of 100 µL with 15 sample replicates. Briefly, the total RNA extraction of TAV infected *N. benthamiana* leaf samples was performed using the 2% CTAB method described by Gambino et al (2008). The concentration of RNA (µg/ µl) was assessed with a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). From each sample, retro-transcription was carried out, starting from 1 µg of RNA, in a 20 µL volume reaction using 0.2 µg/µL random examer primers, 10 mM dNTPs, 0.1 M dithiothreitol (DTT), 2 µL 10x Retro-transcription Buffer, 1 µL M-MuLV (Moloney murine leukemia virus) reverse transcriptase enzyme and water to reach the required volume. The reaction was carried out with the following thermal cycle: 25 °C for 10 min, 37 °C for 45 min, 70 °C for 15 min, and then kept at 4°C.

The targeted fragments of CP-TAV specific gene (494 bp) were amplified using primers containing a linker sequences at their 5'ends. The master mix was prepared in a 25µL volume, containing: 2µL aliquot of TAV- cDNA, 1.25µL of 0.4µM reverse/ forward primer, 5µL of 5X Green GoTaq® Flexi Buffer (Promega), 3µL of 25µM $MgCl_2$, 0.5µL 10µM deoxyribonucleotide triphosphates (dNTPs), 0.125µL of 5U/ µL Taq DNA polymerase enzyme and 11.875µL water to reach the volume of 25µL. The thermocycler conditions used for the PCR constituted an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, and elongation at 72°C for 45 seconds, ending with a longer final elongation step of 7 minutes at 72 °C. All PCR products obtained were visualized by 1% agarose gel electrophoresis in TBE buffer, stained with Midori Green dye and Sharpmass™ 100 molecular marker. The visualized bands were further purified by gel extraction method using the QIAquick® Gel Extraction Kit.

For the second PCR reaction, a specific T7-linker primer (5′-GAGAATTCTAATACGACTCACTATAGGGGATCC-3′) was used to introduce the T7-RNA polymerase promoter sequence appended at both 5′ and 3′ ends. The reaction mixture was prepared in a 50 µL volume, containing: 5µL of the 1st PCR product, 1.25µL of 0.4µM primer pair, 10µL of 5X Green GoTaq® Flexi Buffer (Promega), 6µL of 25µM $MgCl_2$, 1µL 10µM dNTPs, 0.25µL of 5U/ µL Taq DNA polymerase enzyme and 25.25µL water to adjust the volume of 50µL. The PCR conditions were employed same as the 1st PCR, followed by 1% agarose gel electrophoresis visualization using Midori green dye and Sharpmass™ 100 molecular marker.

For the transcription reaction assembly, the second PCR product was used as a template to produce TAV dsRNA. *In vitro* transcription was executed using MEGAscript RNAi Kit (Thermo Fisher Scientific), following the producer's instructions. Before initiating the preparation of reaction mixture, the frozen reagents (T7 Enzyme Mix, 10X T7 Reaction Buffer, 4 ribonucleotide solutions (ATP, GTP, CTP and UTP) were thawed at room temperature and then kept in ice until utilized. The transcription reaction assembly was placed at room temperature containing a total volume of 20 µL. The reaction set up included 2µL of 10X T7 Reaction Buffer, ATP solution, CTP solution, GTP solution, UTP solution and T7 Enzyme Mix, respectively and 8 µL of the 2nd PCR product. After gentle flanking up and down, the tubes were briefly microcentrifuge and maintained at 37 °C for 4 hours.

For gel electrophoresis, 1 µL from 20 µL dsRNA solution was diluted with 99 µL of TE (10 mM Tris, 1mM EDTA). From the diluted mixture, 8 µL was added with 2 µL of 6X loading buffer onto the paraffin and was then loaded into 1% agarose gel (50X TAE) using Sharpmass^{TM} 100 molecular marker. The bands were visualized under the UV light to determine the integrity and efficiency of duplex formation.

The remaining 19 µL from 20 µL dsRNA solution was utilized for the nuclease digestion to eliminate the traces of DNA and ssRNA that did not anneal in the previous steps. For the assembly of RNase digestion reaction, all reagents were kept on ice containing a total volume of 50 µL, by adding Nuclease-free water (22 µL), 10X Digestion Buffer (5 µL), DNase-1 (2 µL), RNase (2 µL), and dsRNA (19 µL), and incubating at 37 °C for 1 hour.

The samples were further purified to remove any leftover traces of proteins, free nucleotides and nucleic acid degradation products by following the MEGAscript RNAi Kit manual instructions. Briefly, the dsRNA binding mix was made in a total volume of 500 µL, by adding 10X Binding Buffer (50 µL), Nuclease-free water (150 µL), 100% Ethanol (250 µL) and dsRNA (50 µL), followed by gentle mixing through up and down pipetting. This dsRNA binding mixture was transferred to the filter cartridge for 2 min centrifugation at 14000rpm, followed by 2 times washing with 500 µL Wash Solution and 2 times recovering of dsRNA with 50 µL Elution Solution (pre-heated at around 95°C) making it a total volume of 100 µL. From the purified dsRNA emersed in Elution solution (total volume 100 µL), the concentration was measured through Qubit high sensitivity assay kit and was analyzed on 1% gel electrophoresis (50X TAE) using Sharpmass^{TM} 100 bp DNA ladder.

After the quantification and gel visualization, the remaining total volume of 1470 µL was utilized for the exogenous application. From the provided volume, a total of 162.1 µL of CP-TAV dsRNA (30 µg) was inoculated on each plant as described in below-mentioned section (2.4.1).

2.4 Exogenous application of dsRNA molecules on N. benthamiana

2.4.1 Experimental setup

The *in vitro* produced dsRNA molecules targeting CP gene of TAV were tested for their efficacy of protection against TAV by exogenous application on three weeks old *N. benthamiana* plants. The treatments were comprised of (1) non-treated as negative control plants (Control-TC); (2) plants inoculated mechanically with CP-TAV dsRNA inoculum comprised of diluted solution buffer at ratio 1:05 (TA-t1) and (3) plants inoculated mechanically with CP-TAV dsRNA inoculum comprised of diluted solution buffer at ratio 1:10 (TA-t2); (4) plants infected mechanically with TAV inoculum only containing solution buffer at dilution factor 1:05 (TB-t1); (5) plants infected mechanically with TAV inoculum only containing solution buffer at dilution factor 1:10 (TB-t2). The number of plants used as biological replicates are listed in Table 1.

The buffer was prepared in a 15 mL total volume by adding 750µL of 0.05M Phosphate-Norit-buffer (pH 7), 0.0168µL of 5mM DIECA, and 30µL of 1mM EDTA. The TAV sap was freshly prepared by grinding the leaves of TAV-infected *N. benthamiana* in previously made buffer (1.5g/1.5 mL). The total volume of solution buffer was prepared by adding TAV sap and buffer with the dilution's ratios 1:5 and 1:10 for each treatment as described in Table 1.
The inoculum mixture was prepared from the total volume of solution buffer, where 16 µL was added with 162.1 µL of CP-TAV dsRNA for TA-t1 and TA-t2 inoculum making a total volume of 178 µL per plant (30 µg per plant). Whereas, for TB-t1 and TB-t2 inoculum, 178 µL from the total volume of solution buffer was taken as it is for each plant and negative control plants were inoculated with only solution buffer (178 µL per plant) (Table 1). The corresponding inoculation mixture (178 µL) for each treatment was applied mechanically to each plant onto the top three fully expanded, carborundum-dusted *N. benthamiana* leaves followed by gentle washing with tap water.

Table 6. List of the all the treatments containing different dilutions and number of plants used as biological replicates

Treatments	Solution Buffer			Inoculum Mixture (per plant)			No. of plants
	TAV sap	Buffer	Total Volume	Solution Buffer	dsRNA	Total Volume	
CP-TAV dsRNA (TA-t1)	200 µL	800 µL	1 mL	16 µL	162 µL	178 µL	4
CP-TAV dsRNA (TA-t2)	100 µL	900 µL	1 mL	16 µL	162 µL	178 µL	4
TAV (TB-t1)	300 µL	1200 µL	1.5 mL	178 µL	-	178 µL	7
TAV (TB-t2)	150 µL	1350 µL	1.5 mL	178 µL	-	178 µL	7
Control-TC	-	1500 µL	-	178 µL	-	178 µL	7

2.4.2 Phenotypic evaluation

After inoculation, the plants were monitored at 6-, 12-, and 18-days post inoculation (dpi) based on the parameters of symptoms severity and plant heights. The symptom severity measurement was observed based on four symptoms: mosaics and blisters, crinkling, leaf distortion, and systemic vein clearing, attributed to a symptom severity class from 0 to 5 (where, 0= no symptom; 1= mild; 2= moderate; 3= severe; 4= extreme; and 5= death of the plant) (Figure 2). These classes were later converted to an infection percentage index (I%I) using the formula presented by Townsend and Heuberger (1943). Whereas, the plant heights were measured from the surface of the soil to the top node in the plant's stem, not taking into account any foliar laminae or flower buds that reached greater heights.

2.5. Statistical data analysis

All the data collected from phenotypic evaluation was subjected to statistical analysis by using R-studio, version 3.6.1 (2019-07-05). In particular, the data obtained from the percentage symptom severity (described in section 3.2.1) were analysed with non-parametric Wilcoxon rank sum test followed by P- values using Bonferroni methods. While plant heights (described in section 3.2.2) were analysed with parametric One-way Anova test followed by Tukey HSD post-hoc multi-comparison test. For both above-mentioned data analyses, non-parametric/ parametric tests were employed based on the degree of normality of the data checked by Shapiro-Wilk's normality test followed by Levene's test for the homogeneity of variances among treatments.

3. Results

3.1 In vitro dsRNA TAV production

The production of dsRNA molecules derived from TAV coat protein (CP) gene was employed on two sequential PCR assays coupled with *in vitro* transcription as described in the section 2.3.2. All PCR products were size evaluated by SharpmassTM 100 bp DNA ladder in a 1% gel electrophoresis.

The results obtained from visualizing first PCR products gave the amplification of CP-gene at around 500bp, but the bands were giving multiple amplification (Figure 2A). Therefore, the gel was cut and purified to optimize the results of first PCR product, that gave the bands a confirmation of examined sequences i.e., 491bp (Figure 2B). From the second PCR assay, very light bands but a little higher in size were appeared as compared to CP-TAV bands, indicating the correct amplification of CP-TAV containing T7-RNA polymerase promoter sequences (Figure 2C). The products of *in vitro* produced CP-dsRNA TAV was subjected to further purification and gave the confirmation of purified dsRNA as shown in the figure 2D. Furthermore, the total quantity of *in vitro* produced dsRNA TAV was found to be 271.95 µg in 1470 µL total volume produced, which was then converted to 30 µg per plant i.e., 162.1 µL per plant.

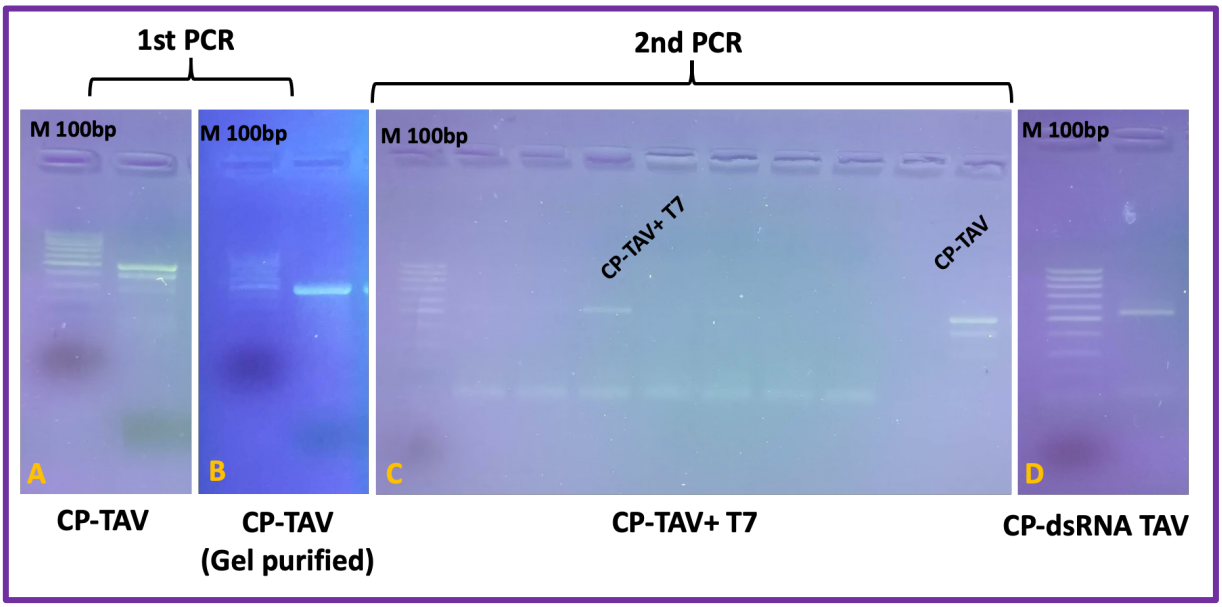

Figure 1. Pictures of gel electrophoresis showing detection of in vitro production of dsRNA molecules with amplified CP of TAV. *(A) the product of the first PCR with amplified CP-TAV carrying linker sequences; (B) band of CP-TAV purified by gel extraction method; (C) the product of the second PCR carrying T7-RNA polymerase promoter sequence; and (D) in vitro produced dsRNA of target CP sequences of TAV. M is a Sharpmass™ 100 bp DNA ladder (Promega Cooperation, USA).*

3.2 Phenotypic evaluation

3.2.1 Symptoms severity

The evaluation of symptoms severity was implemented to describe the influence of the inoculated virus to cause symptoms (mosaics and blisters, crinkling, leaf distortion, and systemic vein clearing) when the plants underwent different treatments (Control-TC as negative control, TA-t1 and TA-t2 indicating dsRNA with diluted solution buffer containing TAV sap at 1:5 and 1:10, TB-t1 and TB-t2 indicating diluted solution buffer containing TAV sap only at 1:5 and 1:10) at different time points after inoculation i.e. 6, 12, and 18 dpi (Figure 2).

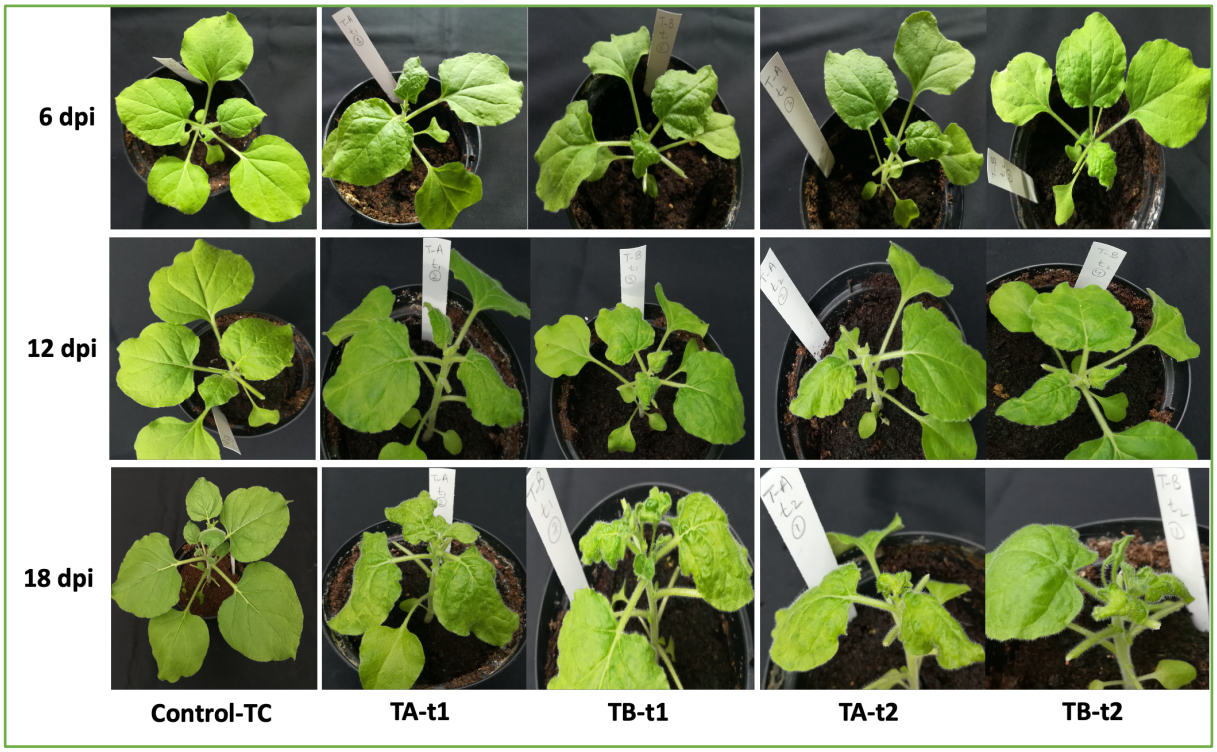

Figure 2. Photographs displaying the symptoms on N. benthamiana plants; *mosaics and blisters, crinkling, leaf distortion, and systemic vein clearing; at 6-, 12-, and 18- days post challenge with TAV and CP-TAV dsRNA molecules applied exogenously. Control-TC are the control treatments displaying no symptoms. TA-t1 and TA-t2 are the treatments attributed to CP-TAV dsRNA inoculum with diluted TAV sap at 1:5 and 1:10, respectively. Whereas, TB-t1 (dilution factor 1:5) and TB-t2 (dilution factor 1:10) treatments are the plants infected with TAV via mechanical inoculation.*

Statistical comparison was made between the different treatments in reference to the prospective timeline (6, 12, and 18 dpi) using the non-parametric Wilcoxon rank sum test followed by P-adjusted values based on Bonferroni methods after checking the normal distribution of data and homogeneity of variances among treatments. The results obtained from

both dilution factor levels (1:5 and 1:10) indicated no statistically significant differences among any treatment, regardless of considered days post inoculation or the combination of treatment and virus (Figure 3). But a slight, non-significant, effect was observed in the symptoms of leaf distortion and mosaics/ blisters at dilution factor 1:10, showing a rise of 40% to 60% in TAV-infected plants (TB-t2), whereas, the CP-TAV dsRNA treated plants (TA-t2) showing the similar percentage level of around 20% (Figure 3).

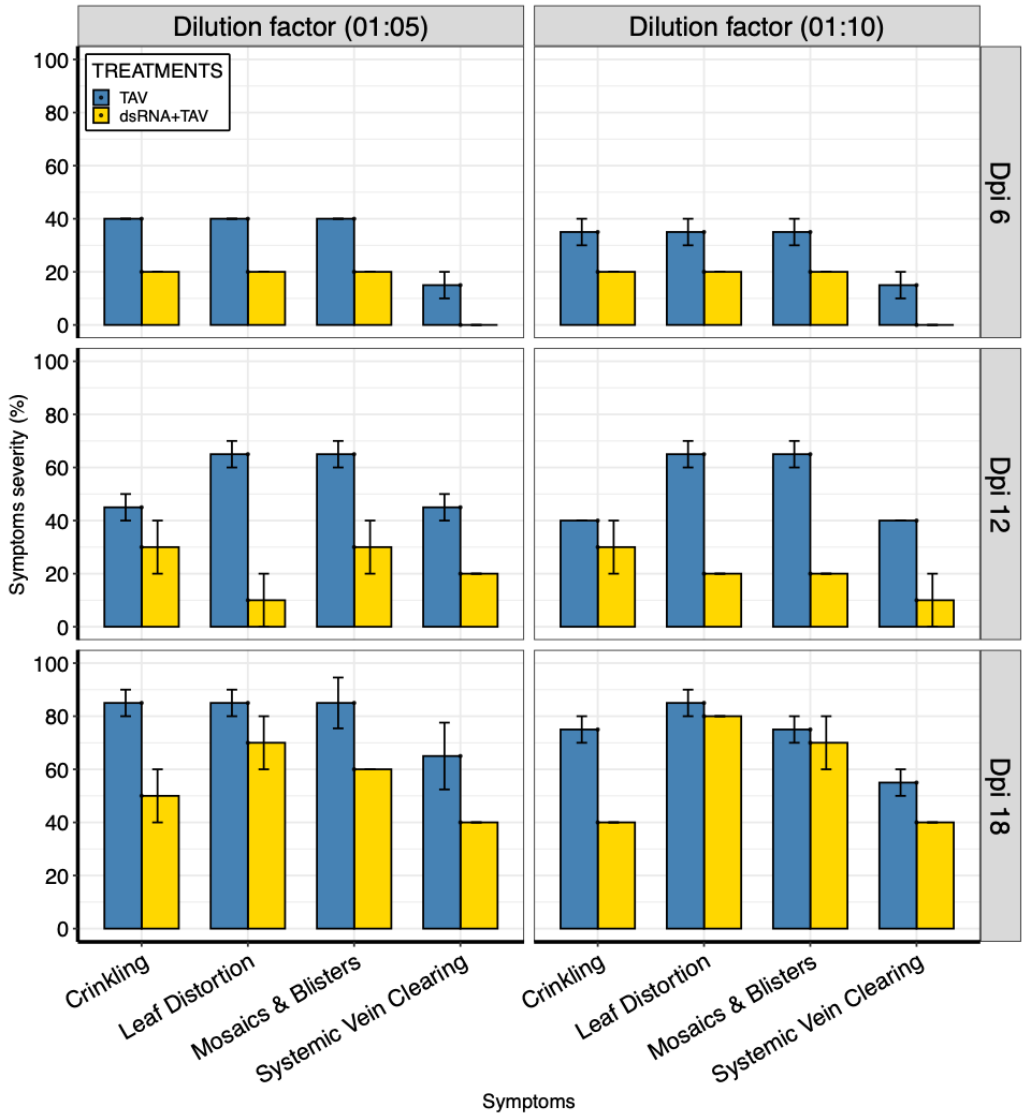

Figure 3. Bar graphs reporting the results of mean values of percentage symptoms severity. *The bar colours represent different treatment: non-treated TAV-infected plants (blue) and CP-TAV dsRNA treated plants (gold). The X-axis represents the symptoms observed at dilution factor (1:5 and 1:10), i.e., crinkling, leaf distortion, mosaics and blisters, and systemic vein clearing. Y-axis represents the symptoms severity scale based on percentage. Error bars indicate standard error whereas no letters indicate statistically non-significant differences ($p < 0.05$) based on non-parametric Wilcoxon rank sum test followed by P-adjusted values based on Bonferroni methods.*

3.2.2 Plant heights

The measurement of plant heights at 6 dpi showed no significant differences among control treatments (Control-TC) and CP-TAV dsRNA treated plants (TA-t1 and TA-t2), but these were grown slightly taller than TAV-infected plants; statistically non-significant to TB-t1 treatment and significant to TB-t2 treatment (Figure 2).

At dpi 12, a slight statistically significant reduction in heights was registered for all the treatments as compared to Control-TC, however, in comparison with TB-t2 treatments, TA-t2 treatments showed slightly greater heights.

The significant difference between TA-t2 and TB-t2 was lost at 18 dpi, by showing similar heights. Whereas, the differences in heights become significantly greater in between treated/ infected plants and control plants. The control treatments gave an increase to plant heights up to 50% as compared to all other treatments (Figure 4).

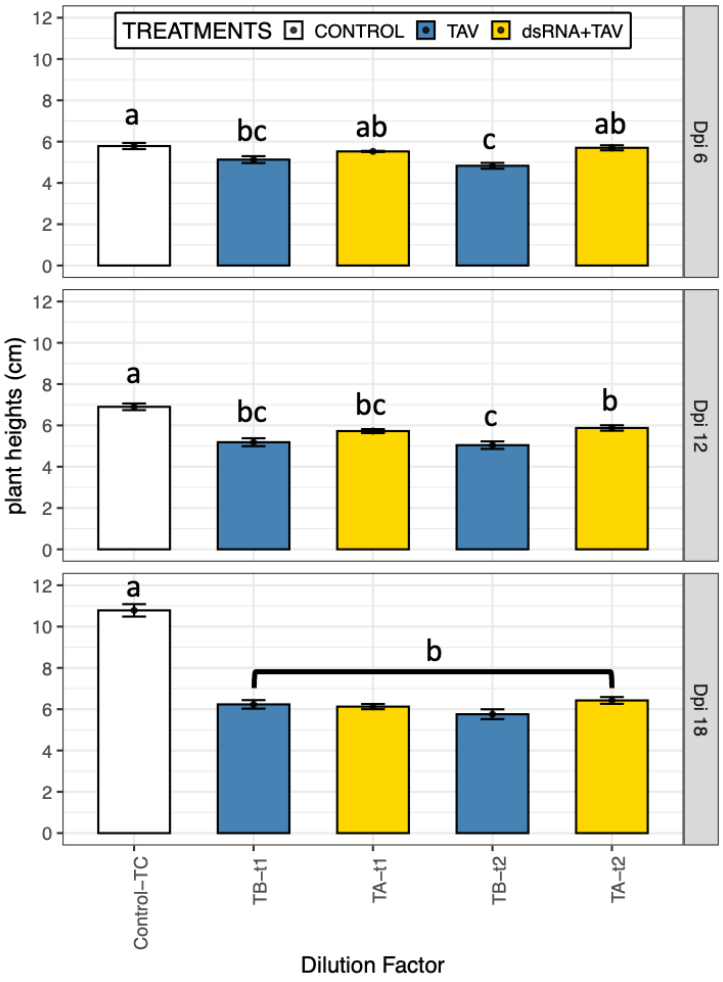

Figure 4. Bar graphs reporting the results of mean of N. benthamiana plants height in cm*. Each bar represents a different treatment: Non-treated healthy control (Control-TC), non-treated TAV-infected plants at dilution 1:5 (TB-t1) and 1:10 (TB-t2), and CP-TAV dsRNA treated plants at dilution 1:5 (TA-t1) and 1:10 (TA-t2), at 6, 12, and 18 dpi. Error bars indicate standard error. Different letters (a-c) indicate statistically significant differences ($p < 0.05$) in the results to One-way Anova (parametric test) followed by Tukey HSD post-hoc multi-comparison test.*

4. Discussion

From the past two decades, RNAi-based biocontrol strategy has offered promising outcomes in providing resistance against plant pathogenic viruses. DsRNA molecules trigger post-transcriptional gene silencing through RNA interference (RNAi) which is highly conserved among eukaryotes. Exogenous application of dsRNA molecules induces resistance not only in transgenic plants but also have been implemented on non-transgenic plants (Carbonell et al., 2008; Konakalla et al., 2016; Mitter et al., 2017b; Tenllado and Dıaz-Ruız, 2001; Worrall et al., 2019).

The present study was conducted using a non-transgenic approach by which exogenously applied dsRNA molecules from TAV CP genes to induce resistance against TAV in *N. benthamiana* plants. For that, the CP targeted DNA fragments were amplified using linker sequences at their 5'ends and were the incorporated with T7 promoter sequences in a two-step PCR assay, followed by in vitro transcription and purification of dsRNA molecules containing CP gene. The *N. benthamiana* plants were mechanically inoculated with CP-TAV dsRNA (mixed in a diluted solution buffer containing TAV sap at ratios 1:5 and 1:10), TAV inoculum (only diluted solution buffer containing TAV sap at ratios 1:5 and 1:10), and a non-treated as negative control (solution containing Norit buffer). The plants were phenotypically examined based on symptoms severity (mosaics and blisters, crinkling, leaf distortion, and systemic vein clearing) and plant heights (in terms of stunted growth) at 6, 12 and 18 dpi, respectively.

The results obtained from symptoms severity showed no significant differences in any of the treatments regardless of considered days post inoculation or dilutions. Inoculation of dsRNA does not significantly reduce symptoms' severity of TAV infection. However, at 12 dpi, there was a slight reduction in symptoms severity among the CP-TAV dsRNA treated plants (TA-t2) but not at significant level. The only significant differences were found in the plant heights at 6 and 12 dpi, where CP-TAV dsRNA treated plants (TA-t2) showed less stunted growth as compared to TAV infected plants (TB-t2). These findings indicate that the *in vitro* produced dsRNA molecules were lacking in efficacy to induce resistance against TAV infection. This less effectivity could be related to the inappropriate concentration of dsRNA inoculum or using of the sequence which is not specific to the particular isolate of inoculated TAV, hence showed incompetency to silence the CP gene expression.

These findings are in agreement with the assessment conducted by Das and Sherif (2020), where the authors quoted numerous studies that have highlighted this apprehension and have shed light on to the factors regarding the inability of dsRNA molecules to induce RNAi

in plants. It includes dose/concentration and size/length of dsRNA, production methodology, delivery techniques, plant organ sensitivity, and instability of dsRNA due to environmental conditions. These factors are not necessarily limited to but may pose an influence in consequently determine the rate of absorption and uptake of dsRNA molecules by plant cells to trigger post-transcriptional gene silencing (Dalakouras et al., 2016; Alexandra S Dubrovina and Kiselev, 2019; Mitter et al., 2017a; Numata et al., 2014).

5. Concluding Remarks

As of now, relatively little is understood about how these factors influences the induction of post-transcriptional gene silencing in plant systems by exogenous application of dsRNA molecules. Hence, the future studies will be implemented to figure out what was the limiting factor that compromised the efficacy of dsRNA inoculation. More precisely, in regard of optimal physical conditions, selection of suitable viral genes, *in vitro* production of dsRNA molecules using different methods (in combination with bacterial endophytes) and with higher length/ size or more specific sequences, along with the exogenous methods. Also, in depth study of understanding the mechanism of plant defense genes expressions will provide better insights on plant protection and improvement of crop productivity.

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Aim# 2
Action against plant pathogenic fungi

1

ePGPBs biocontrol potential against Rhizoctonia solani and Pythium ultimum at pre-harvest stage and its ecological impact on phytomicrobiome

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Passera, A., Vacchini, V., Cocetta, G., Shazhad, G., Arpanahi, A.A., Casati, P., Ferrante, A., Piazza, L., 2020. Towards nutrition-sensitive agriculture: an evaluation of biocontrol effects, nutritional value, and ecological impact of bacterial inoculants. Sci. Tot. Env. 138127. https://doi.org/10.1016/j.scitotenv.2020.138127.

Abstract

Plant protection for phytopathogens is always challenging since the dawn of agriculture. They do not only affect the sustainability of crop production that causes global yield loses but also impacts the ecology of agriculture. Today's agriculture would benefit from applying microbial-based products as they are deemed more sustainable than their synthetic counterparts. This study characterized 3 endophytic plant growth promoting bacteria (ePGPB) strains (*Paenibacillus pasadenensis* strain R16, *Pseudomonas syringae* strain 260-02, *Bacillus amyloliquefaciens* strain CC2) on their biocontrol activity on romaine lettuce plants (*Lactuca sativa*) in greenhouse. The pathogens used in the trials are *Rhizoctonia solani* and *Pythium ultimum*. The obtained results indicate that strain R16 had a significant ability to cause a statistically significant reduction in the symptoms caused by both *P. ultimum* (reduction of 32%) and *R. solani* (reduction of 42%), while the other two strains showed a less efficient biocontrol ability. Whereas, the ecological impact was evaluated by characterizing the bacterial microbiota in bulk soil, rhizosphere, and root in the presence or absence of the inoculants. The composition of the microbiota, analyzed with a Unifrac model to describe beta-diversity, was radically different in the rhizosphere and the root endosphere among treatments, while the bulk soil formed a single cluster regardless of treatment, indicating that the use of these treatments did not have an ecological impact outside of the plant.

Keywords: *endophytic plant growth promoting bacteria (ePGPB), Rhizoctonia solani (RS), Pythium ultimum (PU), green leafy vegetables (GLV), germination percentage (G%), infection percentage index (I%I), microbiota.*

1. Introduction

Plant diseases are a major threat to worldwide food security, causing severe yield loss in all known crop species, and their management is one of the main concerns regarding the sustainability of agriculture: in order to control pathogens and pests, several pesticides are employed, and these can have a high environmental impact (Berg, 2009). The use of more sustainable methods to manage plant diseases is thus a very important step towards making the goals of agricultural sustainability a reality. One of the most promising alternative strategies to the use of synthetic pesticides for a more environmental-friendly control of diseases is the use of bio-control microorganisms (Albouvette et al., 2009). Biocontrol can be defined as the exploitation of organisms, or molecules they produce, capable of reducing or eliminating the damage caused by pathogens, either by direct antagonism or by enhancing the plant's defenses against the pathogens (Junaid et al., 2013). In most cases, biocontrol does not achieve the same level of protection of the crops as synthetic pesticides do and faces the problem of having inconsistent results when used in field scale (Barret et al., 2011), but it is still a promising, more sustainable technique that can be employed in agriculture (Berg, 2009). Despite this, there are several questions regarding the use of biocontrol and its effect on the microbiota of the plant and soil in which they are inoculated. The safety and ecological impact of these inoculants is still a matter of debate in the scientific community (Deising et al., 2017; Koch et al., 2018; Lugtenberg, 2018) and the full extent of the effect of inoculants on non-target organisms is an important point to investigate and define. This is especially true regarding the endophytic communities of the treated plants, in contrast with the effect on the rhizosphere community which has been more extensively investigated (Grosch et al., 2012; Erlacher et al., 2014; Cipriano et al., 2016).

Green leafy vegetables (GLV) are important crops to consider thanks to their high nutritional value and for being consumed mostly raw, keeping intact most of their properties. Among GLV, lettuce (*Lactuca sativa* L.) is one of the most important and common raw edible plants and constitutes a good source of healthy compounds such as polyphenols, carotenoids and vitamins (Becker et al., 2014; Peirez-Loipez et al., 2014). Despite its popularity as a vegetable, lettuce still faces great risks from hard-to-manage diseases caused by soilborne fungal pathogens which can cause devastating losses in field, in particular *Pythium ultimum* and *Rhizoctonia solani* (Van Beneden et al., 2009). The difficulty in managing these pathogens is a limitation both in organic and in conventional farming. For example, *R. solani* was once kept under control by applying methyl bromide, which use was then forbidden because of its

ozone-depleting effect and high toxicity (UNEP, 1999), leaving the farmers with few tools that could be used against this pathogen (Martin, 2003), which can survive in the soil for many years. Since these fumigants are no longer allowed, the pathogens either form sclerotia that can survive in the soil for several years (for *R. solani*) or are often resistant to fungicides (in the case of *P. ultimum*), and *R. solani* is one of the most problematic pathogen to contain in both organic (Termorshiuzen et al., 2006) and integrated farming (Bonanomi et al., 2018), novel tools in the management of these diseases are necessary (Fatouros et al., 2018).

The present study investigated the biocontrol ability of three different endophytic plant growth promoting bacteria (ePGPB) strains on two different fungal, soil-borne pathogens of lettuce, *Pythium ultimum* and *Rhizoctonia solani*. The study did not only evaluate the effect of the inoculated bacteria on their ability to reduce the symptoms induced by the pathogens, but also evaluated the bacterial communities of endophytes in the roots (grown in sterilized or non-sterilized soil), rhizosphere, and bulk soil either non-treated or inoculated with ePGPB strains were described and compared, to define the effect of these treatments on the microbial diversity inside the root tissues.

2. Materials and Methods

2.1. Microbial strains

In this study, three ePGPB strains were used as candidate biocontrol and plant-growth promoting agents: *Paenibacillus pasadenesis* strain R16, which has been already described as a potential antifungal agent in Passera et al. (2017); *Pseudomonas syringae* strain 260-02, which has been already described as a potential biocontrol and plant-growth promoting agent on *Solanaceae* plants in Passera et al. (2019); and *Bacillus amyloliquefaciens* strain CC2. Both strains were cultivated on LB High Salt Agar plates (tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 10 g/L, agar 15 g/L) at 25 °C and were stored in a 20% glycerol solution at −80 °C for long conservation periods.

Two soilborne fungal isolates were used in antagonism assays with bacterial strains: *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; and *Pythium ultimum* Trow, strain DSM 62987 (which will be identified as PU for the rest of the study). The fungal strains were conserved in the fungal culture collection of the Mycology Laboratory at the Department of Agricultural and Environmental Sciences (DiSAA), University of Milan, Italy. The isolates were cultivated on potato dextrose agar (PDA, Difco™) at 20 °C and stored at 4 °C.

2.2. Inoculum with ePGPB strains and plant cultivation

Two weeks old seedlings of romaine lettuce (*Lactuca sativa* L. *var. longifolia*) were inoculated with the bacterial strains by soil drenching: the plants were transplanted in 13 cm in diameter pots containing potting soil and in each pot was added either a suspension of bacterial cells of strain R16, 260-02, or CC2 (10^5 CFU/mL in Ringer's solution) or sterile Ringer's solution for the non-treated control. The potting soil used in these trials was previously sterilized by autoclaving 3 times at intervals of approximately 16 h between each sterilization. The plants were grown in greenhouse at a temperature between 25 °C and 28 °C with 14 h of light per day and were harvested after three weeks from trans-plant for further biochemical and molecular analyses. Each treatment was carried out on 7 plants to obtain biological replicates.

In parallel, a different trial was set up utilizing the same methods, number of plants, and bacterial treatments, but were carried out in soil which was not sterilized. These plants and soil were used for DNA extraction only.

2.3. Biocontrol effect against soilborne pathogens

Biocontrol assays against the fungal pathogens PU and RS were carried out, based on the methods described by Fatouros et al. (2018), with some modifications, in parallel to the cultivation of healthy lettuce plants, and used either plants inoculated with strain R16, 260-02, or CC2, or non-treated controls. For the biocontrol assay, a further positive control was used, treating the potting soil with a *Trichoderma* spp.-based product (indicated as TH from now on) commercially available and indicated for the biocontrol of these soil-borne pathogens on horti-cultural crops; these treatments were performed following the manufacturer's instructions for the utilization of the product. These assays were carried out on 7 plants per treatment.

The fungal inoculums used in these assays was obtained by air drying active cultures of either PU or RS, incubated on pearl millet at 26 °C for 3 weeks. These inoculums were mixed with the sterile potting soil at a concentration of 20 g/kg of soil on the day of the transplant.

The plants were visually examined for the presence of symptoms induced by either PU or RS once a week for a period of 3 weeks after transplant and, at the end of the experiment, the roots were examined as well.

For both pathogens the symptoms included mild to severe leaf and architecture deformation, stunted growth, root damage and, only for RS, crown rot. Root damage could be evaluated only at the end of the trials when the plants were uprooted. The symptoms were evaluated through symptom classes (0: healthy plant, 1: mild leaf deformation, 2: mild leaf and architecture deformation and stunted growth, 3: leaf and architecture deformation and stunted growth, 4: severe leaf and ar-chitecture deformation, stunted growth and root damage, 5: dead plant, caused by crown rot) and these classes were then converted to an infec-tion percentage index (I%I) using the formula proposed by Townsend and Heuberger (1943).

An additional assay was carried out to test the biocontrol effect of strains R16, 260-02, CC2, and TH, used as a positive control, against RS on lettuce seedlings. This assay was carried out using the methods described by Liu et al. (2018), with some modifications. In detail, pathogen inoculum was carried by mixing potting soil with dried RS inoculum one week before sowing, at a concentration of 20 g/kg of soil. Inoculation with strains R16, 260-02, and CC2 was carried out at the same time as sowing by soil drenching using a suspension with a concentration of 10^5 CFU/mL, pouring 1 L every 1.5 kg of soil. For TH the treatment was performed following the manufacturer's instructions for the utilization of the product. Non-treated control (NT) was obtained inoculating the soil with sterile solution, without the bacterial

inoculum. Each treatment was carried out in either soil without RS (Control), or with RS inoculum, in 4 replicates of 50 seeds each. Germination percentage (G%) was eval-uated 5 days after sowing. For this evaluation, seedlings that emerged but died due to damping-off were not considered as successfully germinated.

2.5. Sampling, DNA extraction and 16S sequencing

The following samples were collected for DNA extraction: roots from plants grown in sterile and non-sterile soil, rhizosphere from plants growing in non-sterile soil, and non-sterile soil. For each kind of sample, 7 samples were collected from each treatment (NT, CC2, 260-02, and R16).

For roots, both grown in sterile and non-sterile soil, samples were collected after three weeks from transplant, cleaned from soil and surface sterilized (3′ 70% ethanol, 2′ 5% bleach, 3′ 70% ethanol, 3 washings with sterile water). Starting from 1 g of each of these samples, total nucleic acids were extracted, following the protocol described by Bulgari et al. (2012).

For rhizosphere, the roots were carefully extracted from soil and cleaned from loosely attached bulk soil. The remaining, thin layer of soil clinging to the roots, identified as rhizosphere, was collected in falcon tubes containing sterile water. The suspension was then centrifuged, and the supernatant was discarded. Rhizosphere pellet was then stored at −30 °C until DNA extraction.

For soil samples, a 2-gram aliquot of soil was taken from each pot and stored at −30 °C until DNA extraction. DNA from soil and rhizosphere samples was extracted using the DNeasy PowerSoil kit (QIAGEN), following the manufacturer's instructions. DNA from the sampled roots was sent to an external service (Personal Genomics, Verona (VR), Italy) for sequencing of the hypervariable V3–V4 region of the 16S rRNA gene using a MiSeq1000 sequencer, utilizing a PNA blocker for organellar 16S rDNA amplification (Lundberg et al., 2013). The obtained reads (deposited in EMBL-ENA under acces-sion number PRJEB35767) were analyzed using the QIIME pipeline in order to assign them to OTUs and determine the richness of species in the different samples. Reads that mapped on plant-derived sequences (mitochondria, chloroplasts), and reads with low quality, were filtered out.

2.6. Microbiota analysis

The OTU table obtained from the sequencing analysis was analyzed in R (version 3.6.0) using the R Phyloseq package (McMurdie and Holmes, 2013). A first stage of analysis included

the identification of OTUs that were unique to certain treatments or compartments, opposed to shared or "core" OTUs, considering only OTUs with 10 or more counts per sample type to determine the shared or unique OTUs. These data were visually represented as Venn's diagrams using the online software Venny (Oliveros, 2007–2015). The calculations for alpha- and beta-diversity were carried out as described by Pietrangelo et al. (2018), except as follows: the beta-diversity was calculated exclusively using the weighted Unifrac index, and that 10,000 permutations were used with the adonis function. The composition of the bacterial community, expressed as relative abundance, was defined at the Phylum level and at Family level, with 1% cutoff threshold.

2.7. Statistical analyses

The data obtained from the biocontrol assays (described in Section 2.3) were analyzed as follows: (i) the values obtained for I%I among the different treatments throughout the 3 weeks of observation were compared by performing a general linearized model test, optimized for repeated measures, followed by Tukey's exact post-hoc test ($p < 0.05$); (ii) the values obtained for G% were compared between treatments and pathogen by One-Way ANOVA followed by Bonferroni post-hoc test ($p < 0.05$). The data obtained from the functional health-oriented quality parameters quantification (described in Section 2.4) were analyzed as follows: results obtained in different conditions were compared by a two-way ANOVA followed by Bonferroni multiple comparisons test. Statistics were performed using GraphPad Prism version-6 for Windows, GraphPad Software, La Jolla, California, USA (www.graphpad.com).

3. Results

In the present study the effect of bacterial inoculants on lettuce plants was examined taking into consideration different aspects: bio-control against relevant soilborne pathogens of lettuce (*P. ultimum* and *R. solani*), effects on the physiology and nutritional quality of the leaves, and the effect on the bacterial microbiota associated to the soil and roots of the plants.

Plants were grown in greenhouse conditions, either in healthy soil or in soil experimentally inoculated with the pathogens, to assess the bio-control efficacy of the selected bacterial inoculants in providing biocontrol. Biocontrol efficacy against *R. solani* was also assessed in a different experiment which involved planting seeds of lettuce in healthy soil or soil inoculated with the pathogen, determining how the bacterial inoculants affected germination of the seedlings, a development stage of lettuce that is particularly susceptible to the attack by *R. solani*. All these biocontrol assays included controls that were not treated with any bio-control inoculant, and control that were treated with a commercial bio-control product which uses *Trichoderma* spp. as the biocontrol agent. These results are reported in Section 3.1. Effects on the physiology of the plant and nutritive content was carried out in two stages: during the biocontrol assay, parameters related to photosynthetic efficiency were measured; at the end of the experiment (3 weeks after transplant and inoculation) the leaves were sampled from these plants to quantify chlorophyll content, phenols content, and total carotenoid. The values obtained from these experiments were compared between plants grown with different combinations of pathogens and bacterial inocu-lants to determine if statistically significant differences could be identified, highlighting differences in the physiology of the treated plants. These results are reported in Section 3.2.

Lastly, the effect on the bacterial microbiota was evaluated in four different compartments (bulk soil, rhizosphere, roots grown in sterilized soil, roots grown in non-sterilized soil) related to the healthy plants grown either without inoculation or with one of the bacterial inoculants. The abundance and identity of OTUs was compared among all compartments and treatments; beta-diversity and OTU abundance analyses were carried out to determine which differences could be caused by the treatments. These results are reported in Section 3.3.

3.1. Biocontrol effect against soilborne pathogens

The plants grown in soil inoculated with either *P. ultimum* (PU) or *R. solani* (RS) developed symptoms starting from one week after trans-plant. While the most common symptoms that these pathogens inflict on seedlings (e.g., damping off) were not observed on these plants, symptoms regarding the plant architecture were observed on most plants and, in the case of RS, four plants out of 35 died due to crown rot. Comparison between the I%I in different treatments shows that, for both pathogens, the non-treated plants showed a more severe symptomatology (Fig. 1A and B) compared to the treated plants. In particular, the treatment with strain R16 managed to significantly reduce the I%I for both pathogens, while CC2 and 260-02 reduced the symptom severity but without any significant difference with the non-treated control. Likewise, the treatment with the *Trichoderma*-based product (TH) managed to reduce the symptom severity compared to the non-treated plants, but the difference is not statistically significant.

The results of the germination trial in presence of RS follow the same general trend as the experiment carried out on two-weeks-old plants, except for the treatment with TH which showed an effective biocontrol effect in this trial. The seeds grown without RS in the soil show a high germination percentage (G%), ranging from 62% to 83% and, while there is an increase of G% with the treatments (in particular CC2 and TH), this difference is not statistically significant (Fig. 1C). Seeds grown in the presence of RS instead show differences between the treatments: in the NT seeds, average germination drops from 71% to 26%, the seeds treated with CC2 and 260-02 show an average germination above 30%, while those treated with R16 maintain a higher germination rate of 53%, which is statistically different from those of other treatments with RS, and comparable to that of seeds sowed in soil with-out RS (Fig. 1C). The best result in this assay is obtained by TH, which shows a G% over 70% also in the presence of RS.

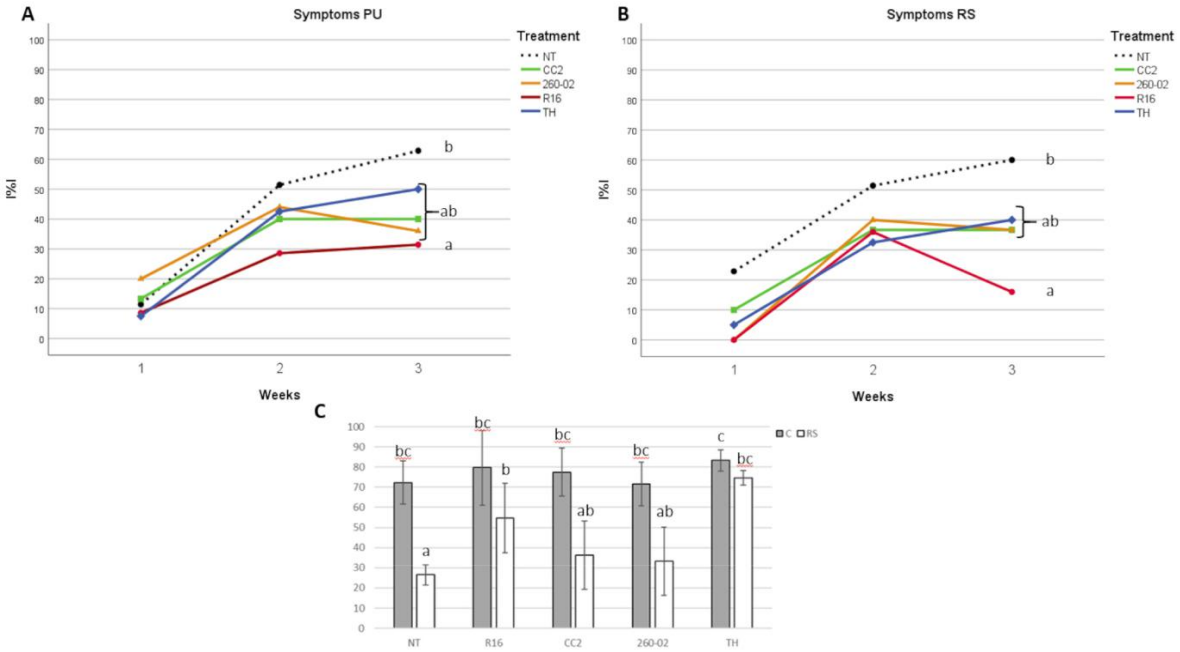

Figure 1. Results of the biocontrol assays. A) *Symptoms observed in plants challenged with Pythium ultimum PU.* ***B)*** *Symptoms observed in plants challenged with Rhizoctonia solani RS. In both graphs, the Y-axis reports the infection percentage index (I%) while the X-axis reports the different weeks of observation. The black dotted line represents the NT plants, the green line with square-shaped dots represent plants treated with strain CC2, the blue line with rhomboid dots represent plants treated with Trichoderma, the yellow line with triangle-shaped dots represent plants treated with strain 260-02, and the red line with circular dots represent plants treated with strain R16. Different letters (a, b) on the right side of the lines indicate statistically significant differences in the results throughout the three weeks of observation, determined by a general linear model, optimized for repeated measures, followed by the Bonferroni post-hoc test (p b 0.05).* ***C)*** *Graph representing the results obtained in the germination assays. The Y-axis reports the germination percentage of the seeds, while the X-axis represents the different treatments. Dark grey bars represent plants grown in healthy soil without RS, while the white bars represent plants grown in soil containing RS. Different letters (a, b, c) on top of the bars indicate statistically significant differences among the results, according to a One-Way ANOVA followed by the Bonferroni post-hoc test (p b 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)*

3.3. Description of bacterial community

Sequencing of partial 16S gene on the surface-sterilized roots (grown in sterilized or non-sterilized soil), rhizosphere, and bulk soil produced, after filtering out organellar sequences, a total of 2.47 million sequences belonging to 7205 different OTUs. Number of sequences and OTUs obtained from each compartments and treatment are reported in Table 1. Of these OTUs, 42 were shared among all compartments and treatments.

Table 7. Sequencing of 16S sequencing, reporting the number of reads and OTUs (expressed as reads - OTUs) produced for each kind of sample analyzed in this study. *Each row indicates a compartment (roots grown in non-sterilized soil, NSR; roots grown in sterilized soil, SR; rhizosphere, RH; bulk soil, S) while each column indicates a different treatment (non-treated, NT; strain 260-02; strain CC2; strain R16).*

	NT	**260-02**	**CC2**	**R16**
NSR	181,906–2131	107,365–1572	106,560–1997	88,911–1525
SR	22,434–1191	14,064–842	10,898–680	13,786–977
RH	301,272–2413	254,580–3357	280,308–3444	247,405–3682
S	217,595–2007	217,721–1997	177,479–2226	226,864–2678

Comparison between the different compartments in the non-treated controls or treated samples showed a high variability among the different compartments: on average, only 5.7% of the OTUs were shared among all 4 examined compartments in each treatment, ranging from 8.4% of shared OTUs between the compartments for NT (Figure 2A) to 3.9% for CC2 (Figure 2D). The highest amount of unique OTUs was registered in the rhizosphere (RH) compartment in all treatments, but it is of note that in the treated plants the amount of OTUs specific to the rhizosphere was around two times higher than in the NT control. On the contrary, roots grown in sterilized soil (SR) showed very few unique OTUs (0.51%). The only two compartments which showed a high level of similarity are rhizosphere (RH) and bulk soil (S), sharing approximately 25% of all the OTUs identified in 3 conditions out of 4 (Figure 2A, B, D), this percentage was lower in plants treated with CC2, only 16%, but this seems to be due to the higher number of OTUs shared not just among rhizosphere and soil, but also in the root endosphere (Figure 2C).

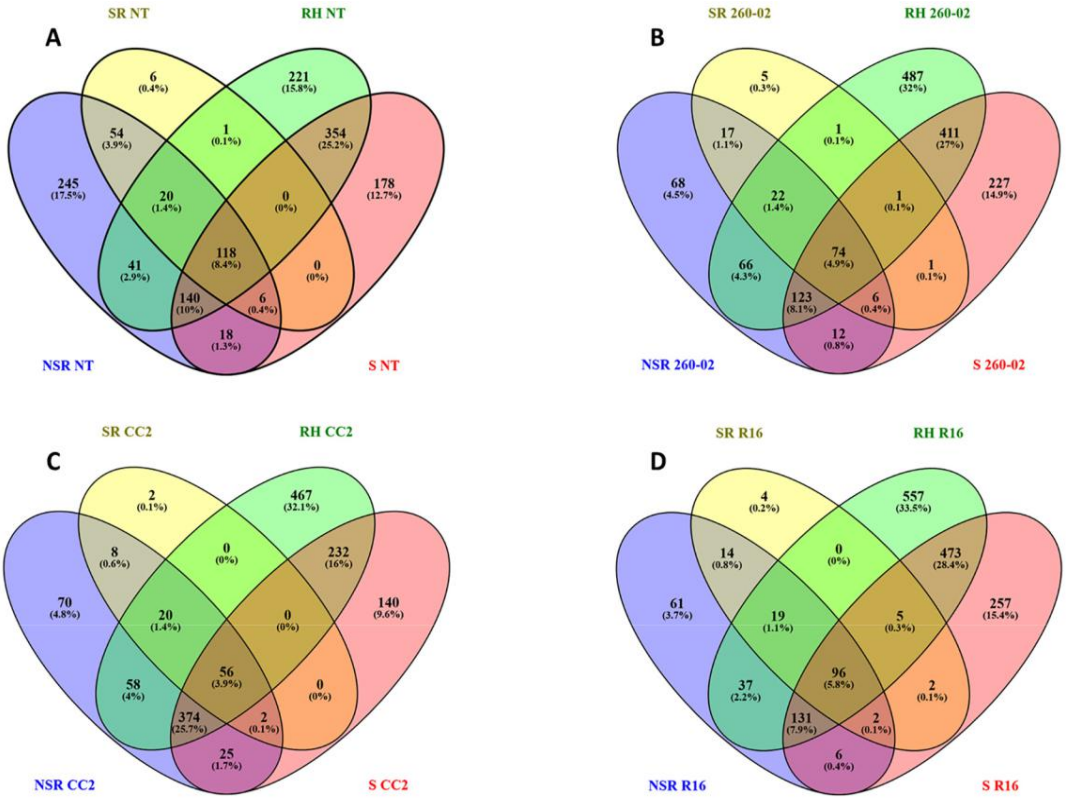

Figure 2. Microbiota: OTU distribution within the same treatments*. Venn diagrams showing the comparative distribution of OTUs in the different compartments analyzed for the same treatment. Each circle is labeled with the compartment (NSR – endosphere of root grown in non-sterilized soil, in blue; SR – endosphere of root grown in sterilized soil, in yellow; RH – rhizosphere, in green; S – soil, in red) and treatment (NT – non-treated; CC2 – inoculated with strain CC2; 260-02 – inoculated with strain 260-02; R16 – inoculated with strain R16).* ***A)*** *Comparison between all four investigated compartments in non-treated plants;* ***B)*** *comparison between all four investigated compartments in plants treated with strain 260-02;* ***C)*** *comparison between all four investigated compartments in plants treated with strain CC2;* ***D)*** *comparison between all four investigated compartments in plants treated with strain R16. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)*

Comparisons between the same compartment among different treatments showed that, regardless of the compartment, the shared OTUs between treatments were slightly above 30%, while the remainder is affected by treatment. In particular, in roots of plants grown in non-sterilized soil (NSR), the highest number of non-core OTUs are those unique to plants treated with strain CC2 (Figure 3A); in roots of plants grown in sterilized soil (SR) the highest number of non-core OTUs was found in non-treated plants (Figure 3B); in rhizosphere samples (RH) the highest number of non-core OTUs were those shared by the three bacterial treatments, but missing in the non-treated control (Figure 3C); in the soil, the highest number of non-core OTUs are those unique to the 260-02 treatment (Figure 3D).

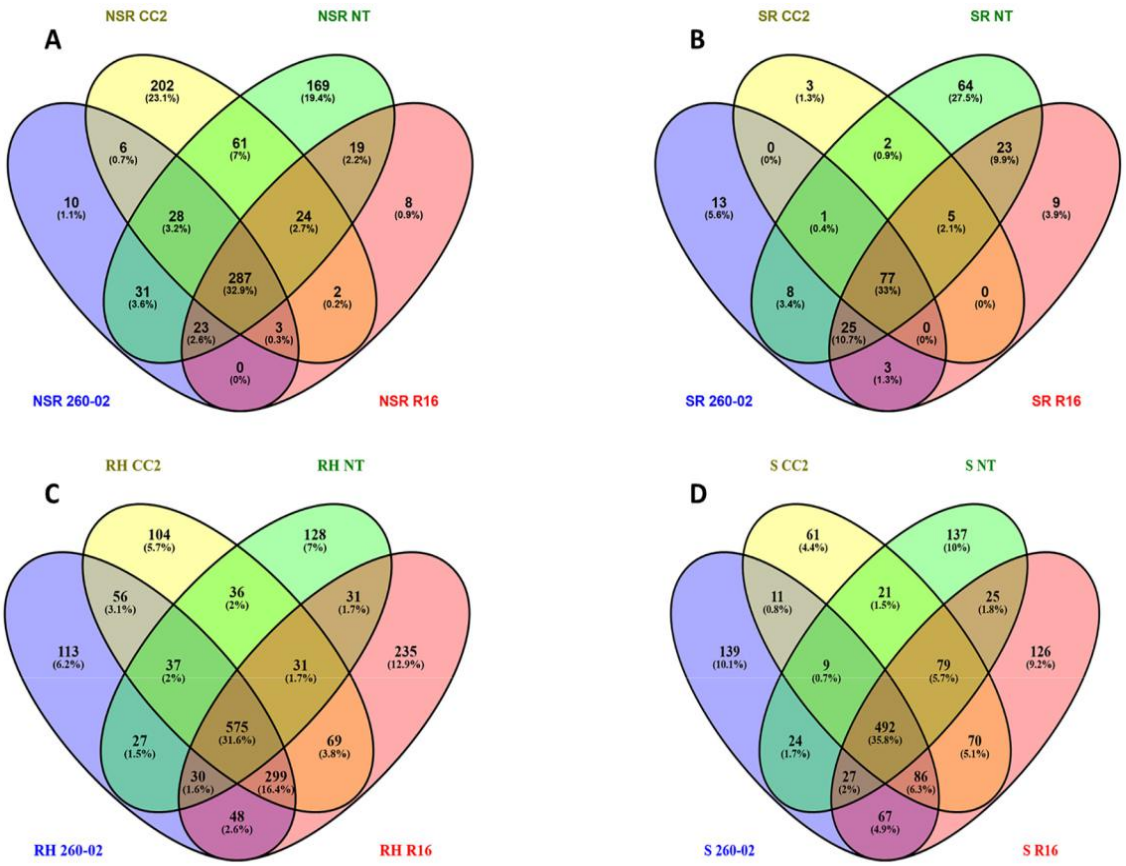

Figure 3. Microbiota: OTU distribution within the same compartment*. Venn diagrams showing the comparative distribution of OTUs in the different treatment for each compartment analyzed. Each circle is labeled with the compartment (NSR – endosphere of root grown in non-sterilized soil; SR – endosphere of root grown in sterilized soil; RH – rhizosphere; S – soil) and treatment (NT – non-treated, in green; CC2 – inoculated with strain CC2, in yellow; 260-02 – inoculated with strain 260-02, in blue; R16 – inoculated with strain R16, in red).* ***A)*** *Comparison between the NSR compartment in all four treatments;* ***B)*** *comparison between the SR compartment in all four treatments;* ***C)*** *comparison between the RH compartment in all four treatments;* ***D)*** *comparison between the S compartment in all four treatments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)*

Analysis of Beta-diversity calculated with the Unifrac model high-light a vast difference between compartments: root endosphere, regardless of soil sterilization, is different from the rhizosphere and soil, which are very similar between them, although clustering separately (Figure 4A). It is interesting to note that, for the non-treated samples, there is no clear separation between bulk soil and rhizosphere, while these two compartments form clearly separate clusters for all the treated samples.

Performing beta-diversity analysis only between the root endosphere samples highlights that there are differences between the microbiota of roots grown in sterilized soil and in non-sterilized soil (Figure 4B). Also, it is possible to see that while the non-treated samples are generally found on the left side of the graph and the treated samples are found on the middle and right side, there is no clear clustering between the different treatments.

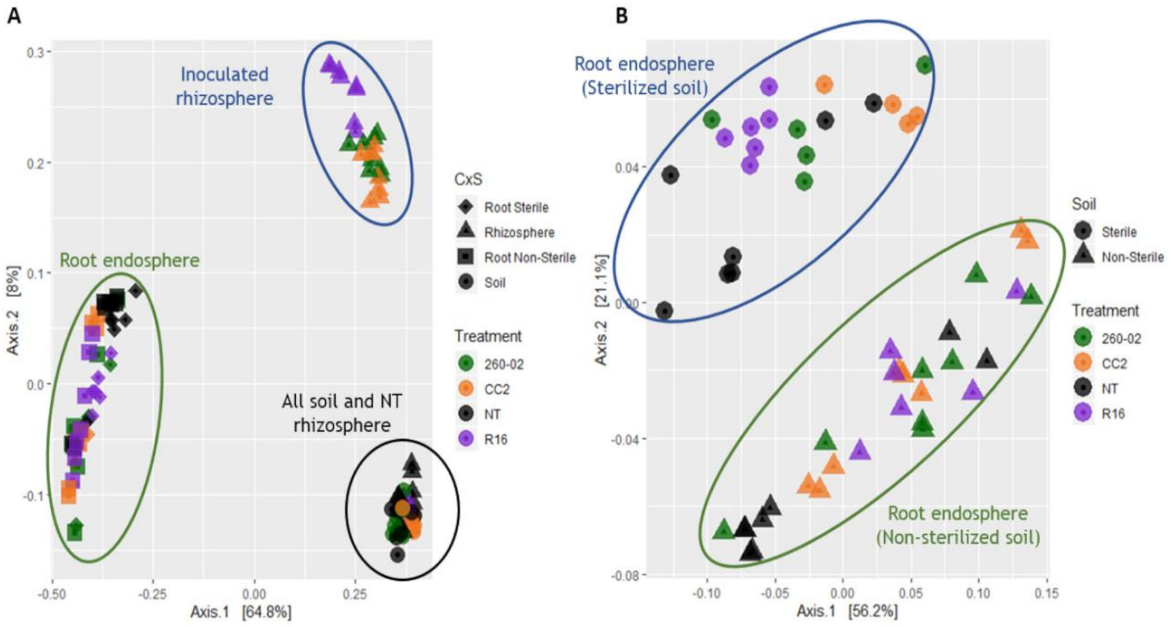

Figure 4. Microbiota: Beta-diversity. *Graphs reporting the distribution of the samples according to beta-diversity calculated with a weighted Unifrac index.* ***A)*** *Graph with all samples; different shape of the markers indicates different compartments and different colors indicate different treatments, as reported in the legend.* ***B)*** *Graph reporting only root endosphere samples; different shape of the markers indicates roots grown in sterilized or non-sterilized soil, different colors indicate different treatments, as reported in the legend. Circles were added to highlight the different clusters of samples.*

Abundance analysis at phylum level shows that all compartments and all treatments are dominated by Proteobacteria, with other relevant phyla being *Verrucomicrobia, Bacteroidetes, Actinobacteria, and Actinobacteria* (Figure 5A). In particular, in root endosphere samples there is a higher abundance of Proteobacteria and lower abundance of the other four aforementioned phyla compared to soil and rhizosphere.

Moving to family level allows to better discriminate between different compartments and treatments. Regarding the *Proteobacteria*, which are highly abundant in all compartments, it can be seen that in the rhizosphere and soil compartments they are mostly composed by *Hyphomicrobiaceae, Caulobacteraceae*, and *Xanthomonadaceae*, while the root endosphere is dominated by *Burkholderiaceae*, but showing also presence of *Xanthomonadaceae*.

The main difference between treated and non-treated samples in the root endosphere and rhizosphere compartments is the family *Oxalobacteraceae*: bacteria of this family are relevantly present in root endosphere of non-treated plants and are absent in the rhizosphere of non-treated plants; on the contrary, they are present only in the rhizosphere of treated plants and absent from the root endosphere of those plants (Figure 5B).

Lastly, in none of the treated samples can be observed an increase in OTUs belonging to the taxonomy of the bacteria used for the inoculation (*Pseudomonadaceae* for 260-02, or *Bacillaceae* for CC2 and R16).

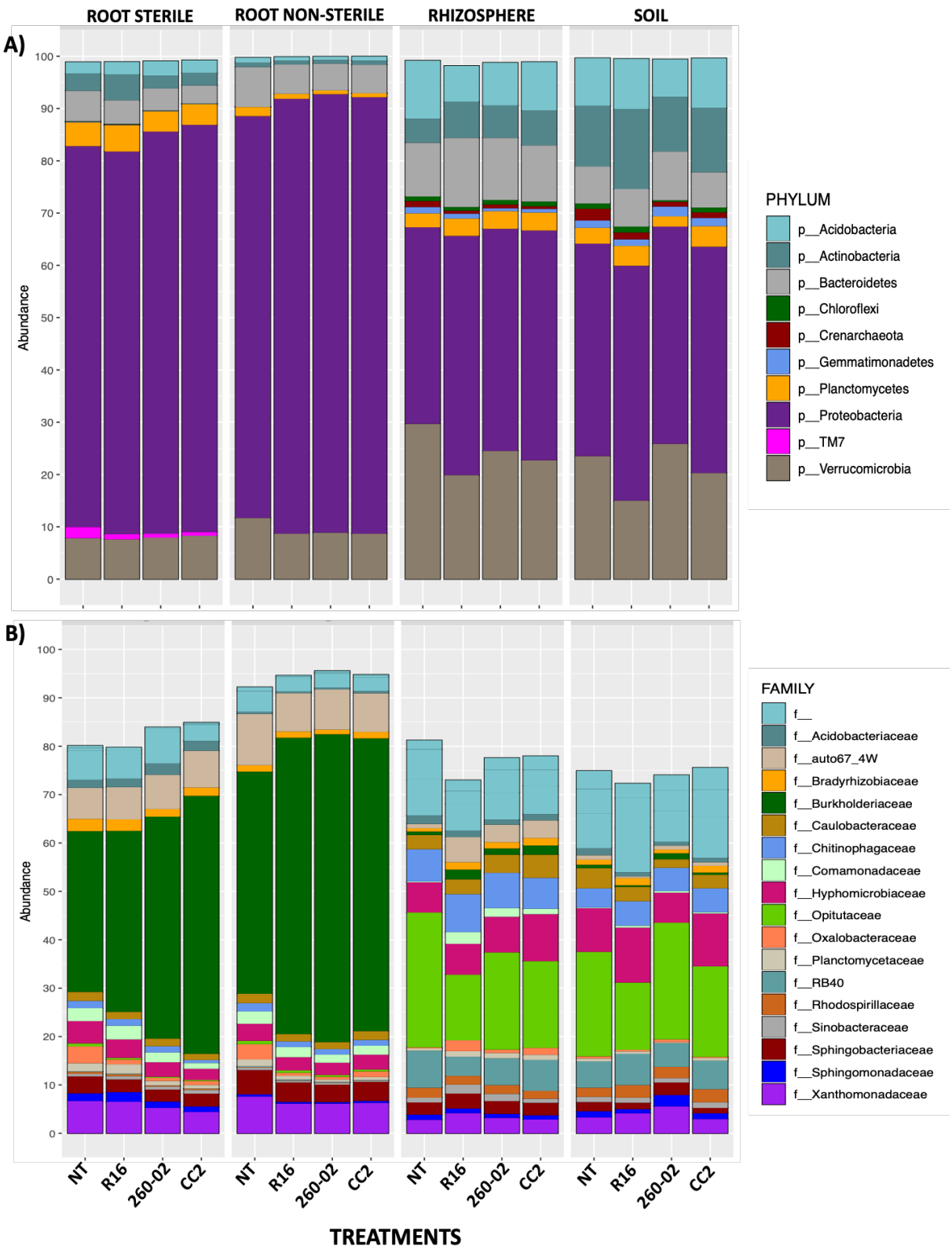

Figure 5. Microbiota: relative abundance*. Graphical representations of the abundance at different taxonomic levels of the microbiota. Stacked bar plots representing the relative abundance of each taxonomical unit (**graph A,** clustered at phylum level; **graph B,** clustered at family level) among the samples. Y-axis reports the relative abundance of the considered taxonomic order, while the X-axis reports the different treatments, divided by compartments in the grid. For ease of interpretation, each graph reports only the most abundant taxonomical groups (cutoff 1%); when a stacked bar does not reach 100%, the missing values belong all to taxonomical groups with abundance lower than the cutoff threshold.*

4. Discussion

While the overall lower environmental impact of biological control compared to synthetic pesticides is well-established (Berg, 2009), in order to confirm to the guidelines of NSA these treatments should also guarantee the quantity and quality of production, while having a minimal impact on the biodiversity found in the agroecosystem.

Regarding the yield, no direct evaluation was carried out in this specific study, but the results obtained in the biocontrol assays can indicate how effective the tested inoculants are in comparison to a commercially available product. Experiments carried out in this study highlighted a positive biocontrol effect of one of the three assayed ePGPB strains, R16, against the fungal pathogens *P. ultimum* and *R. solani*. The other treatments carried out, including a commercial *Trichoderma*-based product, managed to reduce the symptoms induced by both pathogens, although not in a statistically significant way. Results obtained in the seed-germination assay with *R. solani* showed similar results to those obtained on grown seedlings, with strain R16 managing to cause a statistically significant reduction in the symptoms. Interestingly, in this assay also the *Trichoderma*-based product managed to cause a statistically significant reduction in the damage caused by the pathogen, restoring conditions similar to those of healthy plants, suggesting that the development stage of the plant can influence the effect of this product. These results are of particular relevance because, while there are several biocontrol agents (BCA) reported in literature as being able to antagonize either *P. ultimum* or *R. solani*, BCA effective on both are very rare. Only two similar cases are currently reported in literature: *Gliocladium virens* strain G20 (Lumsden and Locke, 1989) and *Paenibacillus alvei* strain K165 (Fatouros et al., 2018). The fact that both strains R16 and K165 belong to the *Paenibacillus* genus might be an indication that further research in broad-range biocontrol strains against soilborne pathogens could become more successful by focusing on bacteria of this genus.

Regarding the effect on bacterial biodiversity, both in the soil and associated to the plant, was analyzed through the 16S amplicon sequencing, producing several interesting results.

The least expected one was that, even though there was no enrichment in OTUs belonging to the bacteria used in the inoculation, the bacterial community of the treated plants was markedly different from that of the non-treated plants. This result indicates that the employed bacteria were either unable to colonize the plants or did so in a transient way, as is often the case with single strains inoculated in a complex microbial community. In spite of this,

the effects on both the plant-associated microbial community and the symptoms caused by the pathogens are relevant. These results suggest that a high rhizosphere competence and ability to colonize the host plant's tissues may not be essential to the development of a beneficial effect, in contrast with previous research that report direct colonization of the host as a necessary step to obtain effective biocontrol (Barret et al., 2011; Ghirardi et al., 2012; Schreiter et al., 2018). The results obtained with three different inoculums show that, while the microbiota associated to the plants faces a shift when exposed to these external bacteria inoculations, the soil microbiota remains largely unaffected, suggesting that the impact on the soil biodiversity is minimal.

One explanation for the reduced symptoms that were recorded could be a direct biocontrol effect against the pathogen, expressed in the early period after the inoculation. Both strains R16 and 260-02 have been reported to have antifungal effect both in *in vitro* and *in vivo* assays (Passera et al., 2017; Passera et al., 2019) and strain CC2 belongs to the *Bacillus amyloliquefaciens* species, for which many strains are known as antifungal agents (Yu et al., 2002; Chowdhury et al., 2013).

Another explanation is that the effect was caused by the shift of microbial community in the rhizosphere and roots. It is possible that the presence of the bacteria either induced directly this shift or caused it through interactions with the plant host. The composition of the rhizosphere microbiota in the treated plants suggests that the biocontrol could be mostly mediated by an activation of the native microbiota since there is a relevant increase of *Oxalobacteraceae*, bacteria previously reported to have an antifungal activity and which abundance has been reported to be positively correlated with soil suppressiveness towards soilborne fungal pathogens (Cretoiu et al., 2013; Li et al., 2015). The facts that (i) bacteria belonging to this family are not found in the bulk soil and in the non-treated rhizosphere, (ii) they are present in the non-treated endosphere, (iii) they are found in the rhizosphere of treated plants, (iv) they are not found the endosphere of treated plants, would suggest the possibility that the treatment could cause these *Oxalobacteraceae* to translocate from the inside of the roots to the rhizosphere, rather than being recruited from the soil.

A third hypothesis on this effect could be made regarding the increase in bacteria belonging to the *Burkholderiaceae* family in the endosphere of treated roots, a phenomenon which is particularly evident for the roots grown in non-sterile soil and treated with strains R16 or 260-02. This family includes the genera *Burkholderia* and *Paraburkholderia* which are well-

known for their plant-beneficial effects (Depoorter et al., 2016), and have recently been described as contributing to soil suppressiveness towards *R. solani* (Carrion et al., 2018).

Lastly, it is interesting to note that the composition of the salad-associated microbiota described in this study is quite different from that reported in some previous publications. For example, Cipriano et al. (2016) describe a lettuce-associated rhizosphere microbiota in which the most abundant genus is *Bacillus*, while in our data the whole *Firmicutes* phylum comes in a negligible amount. This diversity can be explained by having worked on different soils and different lettuce genotypes, stressing once again the role of the environment and of the host's genetic background when describing microbial communities (Bulgarelli et al., 2015). For this reason, studies aimed at describing the effect of host-pathogen-microbiota, especially in the scope of sustainable production, must keep in mind that also the crop genotype, deeper than species level, is an essential variable in determining the success or failure of a biocontrol agent inoculation.

5. Concluding Remarks

In conclusion, biological control agents can represent effective agronomic tools for increasing tolerance to biotic stresses in crops, lowering pesticide applications and reducing the environmental im-pact of cultivation. Our results showed the effectiveness of bacterial inoculants to be used as biocontrol agents for the production of high-quality lettuce following the NSA principles. Positive effect against symptoms induced by soil-borne pathogens was in fact observed, without any adverse effects on plant physiology and quality or on the biodiversity of the soil.

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2

Endophytic plant growth promoting bacteria (ePGPB) induces priming effect through SA and ET/JA mediated pathways to reduce Rhizoctonia solani infection in Lactuca sativa L. (lettuce) at pre-harvest stage

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Abstract

The production of a green leafy vegetable, particularly, lettuce (*Lactuca sativa* L.) is resentfully affected by widespread soil-borne pathogen *Rhizoctonia solani* Kühn (indicated as RS1 in the present study). It causes serious damages worldwide and threatens the agricultural economy. Since the most effective treatment against this pathogen was highly pollutant, researchers have been searching for an efficient and sustainable alternative in biocontrol agents. In this context, the present study was based on evaluating plant growth promotion, biocontrol and priming action of six bacterial strains (*Paenibacillus pasadenensis* R16 strain, *Pseudomonas syringae* 260-02 strain, *Bacillus amyloliquefaciens* strain CC2, *Lysinibacillus fusiformis* strain S4C11, *Paraburkholderia fungorum* strain R8, and *Pantoea agglomerans* 255-7 strain) against *R. solani*. The drench application of ePGPB strains in four different lettuce varieties resulted in highest percentage seed germination for strains R16, 255-7 and S4C11. Strains R16, 255-7 and 260-02 inhibited the RS1 population in rhizosphere and soil. Both results were found to be negatively correlated and indicated a biocontrol effect through antibiosis. To evaluate induction of systemic resistance, gene expression assays were performed on the leaves of plants treated with bacteria and challenged with RS1 at 24 and 48 hpi: an up-regulation of PAL gene in 260-02-treated plants was detected, while PAL and ThlP3 gene up-regulation was observed in 255-7- treated plants, both resulted in SA mediated pathways. The strain R16 triggers PAL and ACCS1 genes, suggesting a co-activation of SA and ET/JA pathways.

Keywords: *Endophytic plant growth promoting bacteria (ePGPB), Rhizoctonia solani, Lactuca sativa L., Antibiosis, priming of plants, Induced systemic resistance (ISR).*

Introduction

Green leafy vegetables, particularly lettuce (*Lactuca sativa* L.), a plant belonging to Asteraceae family, are now recognized as significant part of the daily diet, as they possess an extraordinary attribution to nourishment of human body, in terms of vitamins, minerals and proteins and other numerous bioactive compounds provision. (Armas et al., 2017). The cultivation of this herbaceous plant requires an optimal temperature (16-25 °C) and relative humidity conditions (60-80%) that marks its popularity and cultivation throughout the year worldwide. However, its commercial production is affected by the several diseases at both pre-harvest and post-harvest stage (Claerbout et al., 2019; Vàsquez et al., 2017).

Among the diseases affecting lettuce, bottom rot and damping off, caused by soil-borne fungus *Rhizoctonia solani* [teleomorph, *Thanatephorus cucumeris* (Frank) Donk], is considered as one of the most important pathogen because of its consistency to reside in the soil in the form of dormant propagules (mycelia and sclerotia), and prevails only under the favorable conditions (Singh et al., 2018). Management of this basidiomycete, irrespective of the crop affected, entails the integration of several control measures to limit the number of sclerotia in the soil including chemical, physical and ecological based soil disinfection strategies (Lamichhane et al., 2017). However, the complete pathogen control is quite challenging as *R. solani* inherits aptitude to the saprophytic lifestyle and suitable conditions such as soil moisture, high crop density, temperature conditions and the wide range of hosts that reinforces its survival in the soil (Ajayi-Oyetunde and Bradley, 2018).

Current Integrated Pest Management (IPM) practices have now moved towards using bio-based products that have shown great improvement in the agronomic crops production along with nutritional values upgrade, by synthesizing bioactive molecules and vitamins such as phenolic compounds, ascorbic acid or carotenoids (Cocetta and Ferrante, 2020).

Amid bio-based strategies, use of beneficial microbes as biocontrol agent, is getting more attention due to its dynamic interaction with plants that leads to both physiological and biochemical reprograming of plant defenses (Mauch-Mani et al., 2017). Beneficial microbes produces molecules that a plant receptors recognizes and triggers numerous changes in the host plants and provides with a faster or better coping responses against pathogen invasion (Nawrocka et al., 2018). Moreover, the class of endophytic plant growth promoting bacteria (ePGPB) are considered more suitable as they have advantage over other microbes by being characterized for their endosymbiotic feature, colonization capabilities, and biocontrol

efficiency to a wide spectrum of phytopathogenic invasion particularly, through the mediation of induced systemic resistance (Khare et al., 2018).

From the past few decades, the isolation, characterization and development of products based on utilizing broad spectrum such endophytic microbes as biocontrol agents have been a subject of extensive research. Recently, the studies conducted by Fatouros and colleagues (2018) demonstrated the broad spectrum plant protective capabilities of bacterial biocontrol agent *Paenibacillus alvei* K165 stain against soil-borne pathogens, including *R. solani* in lettuce. Besides acting through antibiosis, K165 stain triggered induced systemic resistance (ISR) in lettuce that mediated the activation of plant defense related genes, hence, conferred the enhanced host protection through priming upon *R. solani* attack. Based on above-mentioned study, similar study was conducted by Aggeli and colleagues (2020), that was aimed to evaluate the efficacy of two strains (*Arthrobacter*-FP15 and *Blastobotrys*-FP12) against *R. solani* and *S. sclerotiorum* in lettuce. The results suggested the early triggering of ET/JA and SA dependent defenses and constitutive triggering of JA and JA/ET dependent defenses mediated by both strains upon *R. solani* and *S. sclerotiorum* in lettuce. Furthermore, in the study of Passera and colleagues (2020), soil drenching of three putative bacterial strains *Paenibacillus pasadenensis* (R16), *Pseudomonas syringae* (260-02) and *Bacillus amyloliquefaciens* (CC2) revealed great biocontrol potential and recorded the reduced symptoms severity on treated *R. solani* infected romaine lettuce, while having negligible effects on both, nutritional quality at the time of harvest as well altering the soil microbial diversity.

Thus, the present study was put forward on testing four different lettuce varieties to assess the broad-spectrum plant protective capabilities of above-mentioned strains including three other strains that are known to have plant growth promotion potential with endophytic lifestyle i.e., *Lysinibacillus fusiformis* (S4C11), *Paraburkholderia fungorum* (R8) and *Pantoea agglomerans* (255-7). The main objectives of this study were to (1) evaluate the plant growth promotion and biocontrol efficacy of ePGPB strains against *R. solani* (*in planta*) on different lettuce varieties, and (2) evaluate the priming action and plant defense responses mediated by ePGPB strains by recording the relative expression of defense related marker genes of SA, and ET/JA, *Pathogenesis-related protein 1* (PR1), *Phenylalanine Ammonia Lyase* (PAL), and *Ethylene Response Factor1* (ERF1), *Thaumatin-like Protein 3* (ThlP3) and *1-AminoCyclopropane-1-Carboxylate Synthase* (ACCS1), upon *R. solani* infected and non-infected Romaine lettuce.

2. Material and Methods

2.1 Plant seeds and microbial strains

2.1.1 Plant seeds

Four seed varieties of *Lactuca sativa* used as test plant in the present study were bought from the local market of Milan (Italy) from the company L'Ortolano: *L. sativa acephala* (Lattuga Rosa- LT0063210200), *L. sativa capitata* (Lattuga Meraviglia d'inverno- LT0004110200), *L. sativa* L. var. *Longifoglia* (Lattuga Romana- LT0040310200) and *L. sativa capitata* (Lattuga la Resistente- LT0002210200).

2.1.2 Pathogen preparation

The soil-borne fungal pathogen *Rhizoctonia solani* (Cooke) Wint, RS1 strain was used in antagonism assay with the ePGPB strains. They were isolated from millet (*Pennisetum glaucum* L.) kernels in 2012 and was then conserved in fungal culture bank of the Mycology Laboratory of Department of Agricultural and Environmental Sciences (DiSAA), University of Milan, Italy. RS1 cultures were grown on potato dextrose agar (PDA; DifcoTM) plates at 20 °C for 7 days and were stored at 4 °C for experimental use.

The soil substrate containing RS1 inoculum was prepared one week before initiating the bioassays using pearl millet as described by Howell (2007) with some modifications. Briefly, the millet seeds (200g) and pre-boiled beans (30g) were mixed together in pre-autoclaved glass-made petri-dish containing 100 mL distilled water. Three actively growing fresh RS1 mycelial plugs (5-10mm diameter) were inoculated to the substrate and incubated for three weeks at 26°C. The mix of millet and *R. solani* was air-dried under laminar flow hood. The culture was macerated into small granules and were mixed thoroughly in sterilized soil. The inoculated soil was stored in plastic bags inside the sterile container at room temperature.

2.1.3 Biocontrol agent preparation

Six strains of candidate endophytic plant growth promoting bacteria (ePGPB), (*Paenibacillus pasadenensis* R16 strain, *Pseudomonas syringae* 260-02 strain, *Bacillus amyloliquefaciens* strain CC2, *Lysinibacillus fusiformis* strain S4C11, *Paraburkholderia fungorum* strain R8 and *Pantoea agglomerans* 255-7 strain), from the culture collection of Department of Agricultural and Environmental Sciences (DiSAA), University of Milan, Italy were used in this study. Except strain CC2, all other bacterial strains were maintained in

Lysogeny broth (LB) supplemented with glycerol in a ratio (7:3) at -80 °C. By following the protocol of Luria and Burrows (1957) formulation, all strains were grown on LB agar (15 g/L Agar, 5 g/L yeast extract, 10 g/L tryptone and 10 g/L sodium chloride) at 24 °C for 48-72 hours.

The liquid suspension of bacterial inoculum was prepared by following the methodology of Passera et al (2019), starting from 16-20 hours incubation of a single actively growing colony of each strain in 3 mL of autoclaved LB with orbital shaking (230 rpm) at 24°C. Another 8 hours incubation was given by transferring 1mL of the culture media in 100mL of autoclaved LB under the same conditions. The bacterial suspension was centrifuged at 4000 rpm for 10 minutes and resuspended in sterile Ringer's solution (Sigma-Aldrich) to adjust the final concentration (10^5 CFU/mL) and the desired volume (1L).

2.2 Evaluation of % seed germination of lettuce variety

The bioassay of % seed germination was carried out in two sets of frameworks in the greenhouse under control conditions of 14 h photoperiod at 25-28°C temperature. First setup constituted the evaluation of the strains R16, 260-02 and CC2 efficacy on the varieties other than *Lattuga Romana* as the results of these strains on *Lattuga Romana* has already been discussed by Passera et al (2020), presented as Chapter 1 of Aim 2 of this thesis. The second setup represented the bioassay for evaluating strains S4C11, R8 and 255-7 on all four selected varieties of lettuce.

Both setups involved four treatment groups that contains: (1) non-treated soil as a negative control (NT); (2) RS1-infected soil as a positive control (RS1); (3) ePGPB strains inoculation on non-infected soil (BCAs: R16, 260-02, CC2, S4C11, R8, 255-7); (4) ePGPB strains inoculation on RS1-infected soil (RS1 + BCAs).

For each lettuce variety, a total of 10 seeds per row were sowed in soil potting trays containing 1.5 kg of soil, irrespective of treatment groups followed by three biological replicates. At the same time, 1L of bacterial suspension was soil drenched in soil potting trays containing RS1-infected and non-infected soil, respectively. Whereas, the other soil potting trays containing 1.5kg of soil were designated to negative (NT) and positive control (RS1) groups by inoculating with 1L of Ringer's solution only. The number of successful germinated seedlings were counted at 5 days post inoculation (dpi) and were transformed to a percentage of germination (G%), however, the seedlings that died after emergence and showed symptoms of damping-off were not considered for the evaluation.

2.3 Determination of RS1 rhizosphere soil and soil population

After evaluating % seed germination bioassay, only one lettuce variety *L. sativa* L. var. *longifolia* (*Lattuga Romana*) was selected for further experiments. In this bioassay, the efficacy of bacterial strains to colonize and inhibit directly the rhizosphere and soil population of RS1-infected *Lattuga Romana* were evaluated at 7 dpi containing treatment groups (NT, RS1, RS1 + BCAs). Bulk sampling of rhizosphere soil (the soil containing root microbiome) and the soil (collected from the vicinity of the plant) was done by plucking few lettuce seedlings and were transferred to five sterilized falcon tubes as five replicates per treatment. The population estimation was performed using the methodology of serial dilution and plating described by Fatorous et al (2018) with some modification. Briefly, 1g of collected samples (rhizosphere soil and soil) were immersed in the falcon tubes containing 50mM of sodium phosphate buffer (pH 7, 0.02% Tween 20) and incubated for 45min on orbital shaking at 30 °C. Five 10-folds dilutions (up to 10^6 cfu/g) were made in sterile buffer and were shaken softly with hands for few seconds to make a homogenous suspension. The suspension of 100 µL was spread evenly onto the PDA plates supplemented with the cocktail of antibiotics (50 µL/mL Kanamycin, 50mg/L Ampicillin, 10mg/L Tetracycline Hydrochloride and 0.2% 2mg/L Dicloran) to inhibit the bacterial growth. The plates were preserved using paraffin to avoid contamination and incubated for 48h at 30 °C in order to determine the number of RS1 colonies using colony forming unit per gram (CFU/g).

2.4 Evaluation of plant defense genes

2.4.1 In planta bioassay and sampling

For the evaluation of plant defense related genes, *in planta* bioassay was conducted comprising of five treatment groups (NT, BCAs, RS1, RS1 + BCAs) with five biological replicates per treatment. Three weeks old *Lattuga Romana* seedlings were transplanted to the RS1-infected and non-infected soil pots (13 cm), respectively. On the same day of transplantation, treatment groups (BCAs and RS1 + BCAs) were soil drenched with bacterial suspension (50mL, 10^5 CFU/mL), whereas, the control groups (NT and RS1) were inoculated with 50mL Ringer solution in the pots containing soil. After 24h and 48h of inoculation, 0.5 g of leaf tissues from each treatment were harvested and stored immediately at -80°C for RNA isolation.

2.4.2 RNA isolation, cDNA synthesis and quantitative Real-time PCR

A protocol of 2% CTAB method was used to isolate total RNA from the frozen leaf tissues of each treatment as described by Gambino et al (2008). The RNA quality and quantity were measured on NanoDrop spectrophotometer. The transcripts of first stand cDNA were synthesized using two steps retro-transcription polymerase chain reaction (RT-PCR) in a 20 µL of total volume. Briefly, 1µg of total RNA, 0.5µL random examer primers (0.2 µg/µL), 1 µL dNTPs (10 mM) and nuclease free water were added to the RNA mixture tube to reach 11µL of volume. After the denaturation of RNA (90°C for 5 min followed by the incubation at 4°C), a second reaction mixture of 2µL Retro-transcription Buffer (10x), 4µL $MgCl_2$ (25mM), 2µL Dithiothreitol (DTT, 0.1 M) and 1 µL M-MuLV (Moloney murine leukemia virus) reverse transcriptase enzyme were added to the tubes. The thermal cycler was set up on following cycles: 25 °C for 10 min, 37 °C for 60 min, 70 °C for 5 min, and then kept at 4°C.

The relative quantification of the defense related genes was performed using real-time PCR assays (qRT-PCR) in a StepOnePlus™ thermocycler (Applied Biosystems™). It was conducted using the reaction mixture of 1x Power SYBR® Green PCR Master Mix, 400 nM of each primer containing gene of interest, 2 µL aliquot of cDNA, and nuclease free water to get the final volume of 20 µL. Each gene of interest was calculated using the average threshold cycle (Ct) of three biological replicates per treatment. The data of Ct values were normalized by using endogenous plant gene *Adenosine Phosphoribosyl Transferase 1* (APT1) as internal standards of $2^{-\Delta\Delta CT}$ methods as described by Livak and Schmittgenin (2001).

For the relative quantification assay, two defense related genes *Pathogenesis-related protein 1* (PR1), *Phenylalanine Ammonia Lyase* (PAL), and *Ethylene Response Factor1* (ERF1) were selected from the study of De Cremer et al (2013), whereas, *Thaumatin-like Protein 3* (ThlP3) and *1-AminoCyclopropane-1-Carboxylate Synthase* (ACCS1) gene was used from the study of Safavi et al (2012) and Albano and Macfie (2016), respectivley. The sequences of all the primer pairs of selected genes are referenced in the table 1.

Table 8. List of all the primers used for the relative quantification of gene expression analysis performed by qPCR assays

Lettuce ID	Primers for the Gene Expression			
	Primer Pair	**Sequence 5 '- 3'**	**Fragment (bp)**	**Bibliography**
Lsa039252.1	APT1 - forward APT1 - reverse	CTGTACAAGAAGGAGAACGAGC ACGAGCACATACAGTGGCTT	184	Argyris et al., 2008
Lsa018589.1	PR1 - forward PR1 - reverse	ATGGGACAGTCGTGTGGCTAGTTT TGTTCACAGCATCTACACCGGTCA	190	(De Cremer et al., 2013)
Lsa044239.1	PAL - forward PAL - reverse	TGGCCCCACCGGAGAAGTCC GGAAGCCATCCCGGACCCCA	136	(De Cremer et al., 2013)
Lsa003059.1	ACCS1 - forward ACCS1 - reverse	TGAGCGGTGGAGCCACTGGA GGGGTGGGCACCAAGAAGGC	82	(Albano and Macfie, 2016)
Lsa016859.1	ERF1 - forward ERF1 - reverse	TCGCCGGTGATGTCCAGTTATCAA TGTTTCCCTCTCTGCTGGTTCACA	83	(De Cremer et al., 2013)
Lsa000649.1	ThlP3 - forward ThlP3 - reverse	GATGAGGTTTCAGCAGCCGTGTTT TAATGAGAATCCGCCGTCGCCTAA	114	(Safavi et al., 2012)

3. Results

3.1 Evaluation of % germination of lettuce variety

The measurements taken at 5 dpi to evaluate % viable seed germination of lettuce varieties challenged with or without RS1 and treated or non-treated with bacterial strains showed interesting results.

Seeds of all lettuce varieties grown in the RS1-infected soil showed great significant reduction in germination as compared to NT soil and dropped off the germination by an overall average from 82.5% to 50.5% (Fig. 1). Furthermore, the soil treatment with all bacterial strains, irrespective of RS1-infected and non-infected treatments, showed either similar trend of viable seed germination rate or higher as compared to control treatments of all the lettuce varieties. Such as, the strain 260-02, although did not provide statistically different results in any treated groups, it upheld the comparable seed germination rate to that of bacterial strains treated and non-treated seeds variety (Fig. 1A). Besides, an unexpected result was observed by the strain CC2 treated *Lattuga Rosa*, that showed the lower percentage of viable seed germination in both non-infected and RS1-infected soil as compared to NT and RS1-infected soil, respectively (Fig. 1A).

Among the non-infected groups, strain R16 managed to produce highest number of viable seeds germination of *Lattuga Resistente* variety i.e., 100% as compared to non-treated control that was able to germinate around 80% of viable seeds (Fig. 1A). The second highest score was recorded for the strain 255-7 in the non-infected group, that is around 90%, in the three out of four lettuce varieties (*Lattuga Inverno*, *Lattuga Resistente* and *Lattuga Rosa*), while, the non-treated control germinated around 80% of viable seeds. (Fig. 1B).

In comparison to all seed varieties grown in RS1 infected soil, it is promising to note that the strain R16 showed consistent results in *Lattuga Rosa* by germinating around 80% of viable seeds, when comparing the non-treated control with non-treated RS1-infected control, that dropped the seed germination count from around 90% to around 60% (Fig. 1A). Similar trend was observed by the strain 255-7 that was inoculated in *Lattuga Rosa and Lattuga Resistente,* and presented with around 90% consistent growth promotion, while both NT and RS1-infected control groups were shown reduction in seed germination rate from around 70% to 50%. Additionally, the strain 255-7 displayed statistically significant difference among the treated and non-treated RS1-infected groups with higher seed germination rate in *Lattuga Inverno*, *Lattuga Romana* as well. Another promising statistically significant higher

germination rate was observed in S4C11-treated RS1-infected soil potting trays containing *Lattuga Romana* (Fig. 1B).

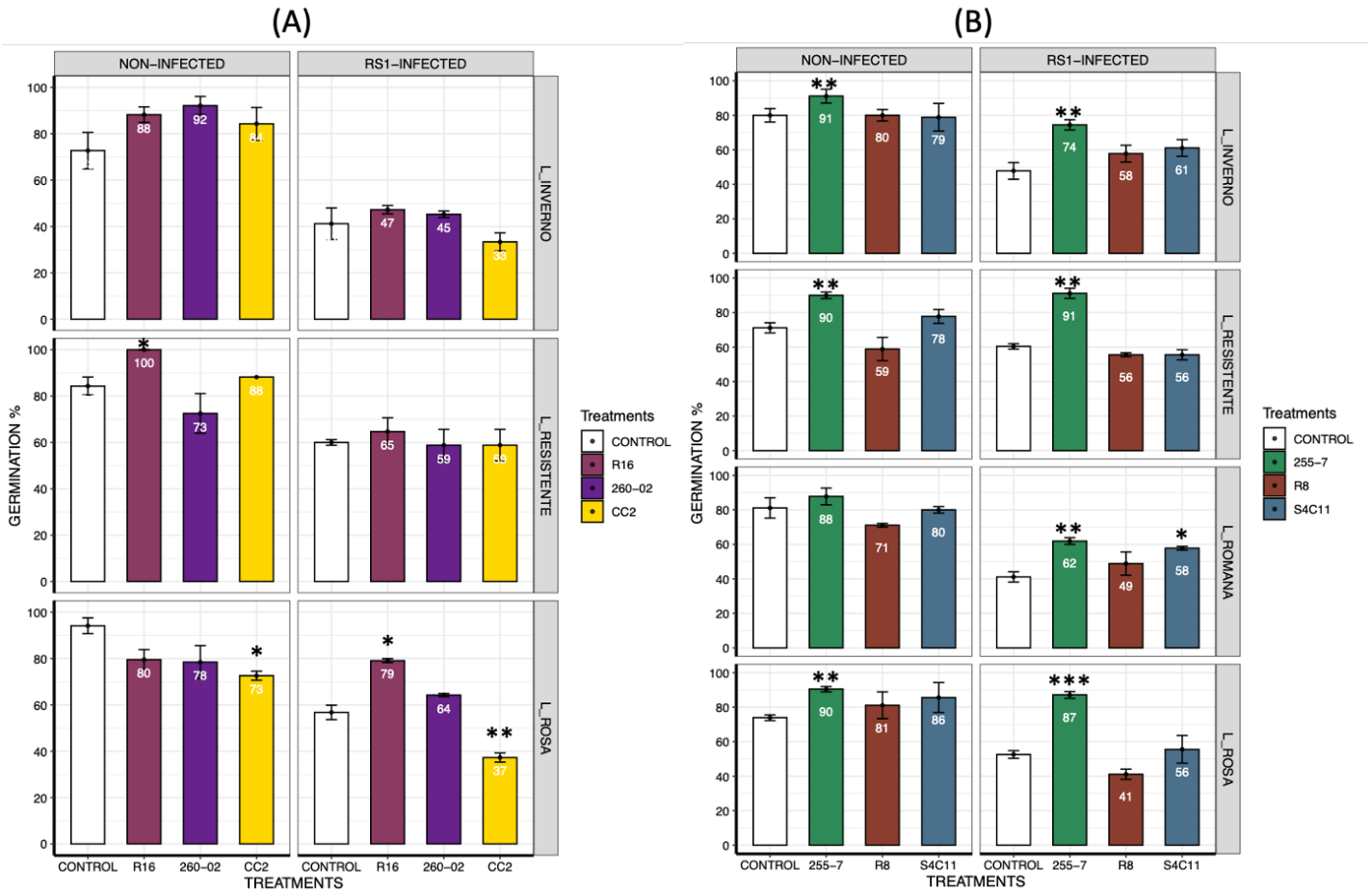

Figure 1. Bar graphs showing the results of % germination assays measured at 5 dpi. (A) *Effect of strains R16, 260-02 and CC2 on the RS1 infected and non-infected soil containing lettuce seeds variety except Lattuga Romana.* ***(B)*** *Effect of strains 255-7, R8 and S4C11 on the RS1 infected and non-infected soil containing all four lettuce seeds variety. Control represents for negative control non-treated group belong to the soil that were previously RS1-infected and non-infected. Values are means of 10 seeds per treatment ± standard errors (SE) in triplicates. Asterisks indicate significant differences among the results according to the t-test followed by Bonferroni methods compared with the control treatments of each group (* for p < 0.05, ** for p < 0.005, *** for p <0.001, **** for p =0.000).*

3.2 Determination of RS1 rhizosphere soil and soil population

This bioassay was performed based on the findings of % germination assay, from which only three ePGPB strains (R16, 260-02 and 255-7), treated on to the lettuce variety *Lattuga Romana,* were selected for carrying out further experiments. This bioassay was conducted by serial dilution and planting as described in the (section 2.3) and have shown promising results.

Overall, the data extracted at 7 dpi from the log10 values of 10^4 cfu/g have shown the highest number of RS1 population found in the RS1-infected rhizosphere ($1.35*10^4$ cfu/g) followed by RS1-infected soil ($1.08*10^4$ cfu/g) samples (Fig. 2). Whereas, the RS1 population observed in bacterial treated rhizosphere soil and soil samples were in much lower abundance. According to percentage reduction estimation (data not shown), the most promising colonizing assurance and direct antagonistic effect was displayed by the strain R16, that reached to a maximum level of RS1 population reduction (33.3%), followed by the strain 255-7 (30.4%) and strain 260-02 (20.7%) in RS1 infected rhizosphere soil. Whereas, the second interesting result was exhibited by the strain 255-7, that demonstrated not as great as in rhizosphere soil, but a significant percentage reduction (23.1%) followed by the strain R16 (20.4%) and strain 260-02 (14.8%) in the RS1 infected soil.

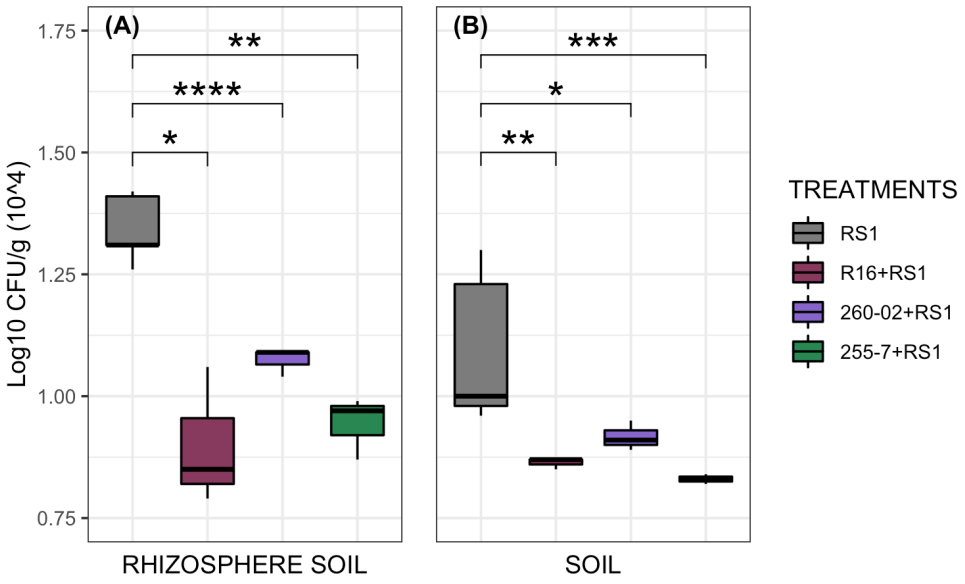

Figure 2. Boxplots reporting the results of RS1 population collected from (A) rhizosphere soil and (B) soil; treated with/ without bacterial strains containing Lattuga Romana, challenged with RS1 at 7dpi. *The Y-axis represents the Log10 transformed values of 10^4 CFU/g RS1 population, while the X-axis represents the different treatments. Asterisks indicate significant differences among the results according to the t-test compared with RS1-infected non-treated groups (* for p < 0.05, ** for p < 0.005, *** for p <0.001, **** for p =0.000).*

3.3 Effect of % seed germination on RS1 rhizosphere soil and soil population

To demonstrate the correlation effect of the *Lattuga Romana* seed germination amended with bacterial strains in response to RS1 infection and its population present in rhizosphere soil and soil, a ggscatter plot with a linear regression analysis was presented in figure 3.

The results obtained from the data demonstrated that there was a statistically significant negative correlation between the % of viable *Lattuga Romana* seed germination and the RS1 population (CFU/g at 10^4) observed in both the rhizosphere soil (P= 0.02, R=-0.46) and the soil (P= 0.04, R=-0.42) of the same plant (Fig. 3). In both sample types, the RS1 treatments tend to show increment in cfu/g population and consequently lowered the rate of % seed germination. On the contrary, strains R16 and 255-7 managed to reduce the RS1 population and promoted the seed germination. The strain 260-02 showed the reduction in RS1 population but did not endure to significantly promote the seed germination (Fig. 3).

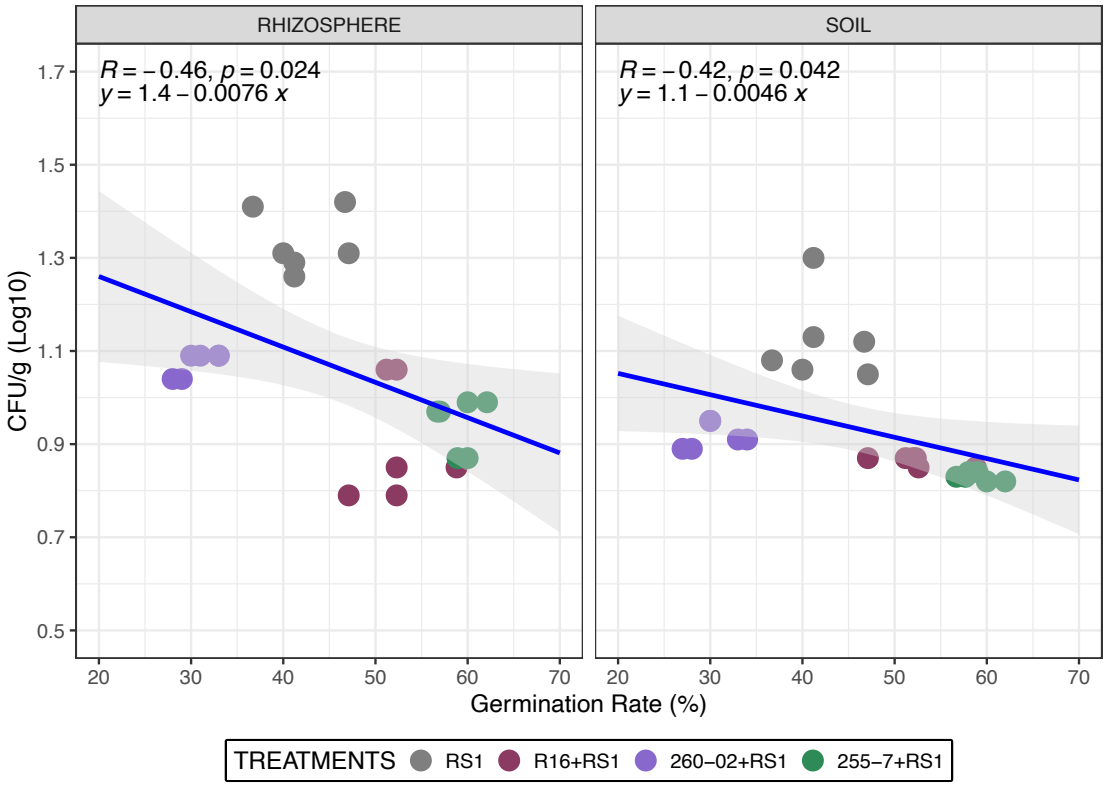

Figure 3. Linear regression analysis representing the effect of RS1 infection in Romaine lettuce either treated with or without bacterial strains (R16, 260-02 and 255-7). *The samples were collected from* ***(A)*** *rhizosphere soil and* ***(B)*** *soil. Y-axis represents the Log10 transformed values of 10^4 CFU/g RS1 population, whereas the X-axis represents percentage of viable seed germination. R-values shows the coefficient of correlation of the model, y-values represents the regression slope intercept and p-values presents the significant difference (p < 0.05) between each treatment.*

3.4 Expression of plant defense genes

The hypothesis that the candidate ePGPB strains (R16, 260-02 and 255-7) alone or upon RS1 infection in *Lattuga Romana* seedlings might have an involvement in inducing plant defense responses. To examine in depth, five plant defense genes that are involved in salicylic acid (SA) and Jasmonic acid (JA)/ Ethylene (ET) regulation (PR1, ERF1, ACCS1, PAL and ThlP3 genes) were analyzed by qRT-PCR.

According to the results obtained from the log10 transformed $2^{-\Delta\Delta CT}$ values, the up-regulation was observed in RS1-infected leaves that followed the activation of highest expression levels of all the examined genes at 24hpi and 48hpi respectively (Fig. 4).

The presence of candidate bacterial strains alone or in RS1 infected plants demonstrated downregulation of PR1 gene at both 24hpi and 48hpi except in the 260-02 + RS1 treated plants that showed an upregulation at 48hpi (Fig. 4A).

Furthermore, 255-7 treated plants showed an upregulation of ERF1 transcript levels at 24hpi while the other treatments followed the same trend of downregulation as PR1 when compared to the RS1-infected plants. However, at 48hpi, the ERF1 levels of all treatments became non-significant, excluding the R16 treatment which was continuously downregulating the ERF1 gene (Fig. 4B).

In case of ACCS1 gene, downregulated transcript levels were observed only at 24hpi among the treatments R16, 255-7, 260-02 + RS1 and 255-7 + RS1, whereas, rest of the treatments at 24hpi and 48hpi respectively did not show significant differences as compared to RS1-infected plants (Fig. 4C).

On the other hand, PAL gene at 24hpi showed downregulation in the R16 treated and 255-7 + RS1 treated plants, however, there was a slight upregulation observed in the 260-02, 255-7 and 260-02 + RS1 treated plants. Moreover, the R16 + RS1 treatment at 24hpi showed insignificant upregulation of PAL transcripts as compared to RS1 treated plant. And then, at 48hpi, it displayed downstream of PAL gene along with R16 and 260-02 treated plants, whereas, all other treatments were upregulated by following the same trend of RS1 treatments (Fig. 4D).

For the gene ThlP3, a statistically significant decrease in the transcript abundance can be seen for the plants treated with all bacterial strains at 24 hpi. Whereas, the strain 260-02 in the absence of RS1 and R16 strain with or without the presence of RS1 pathogen showed downregulation at 48hpi while the other treatments were not significantly different from RS1 treatment (Fig. 4E).

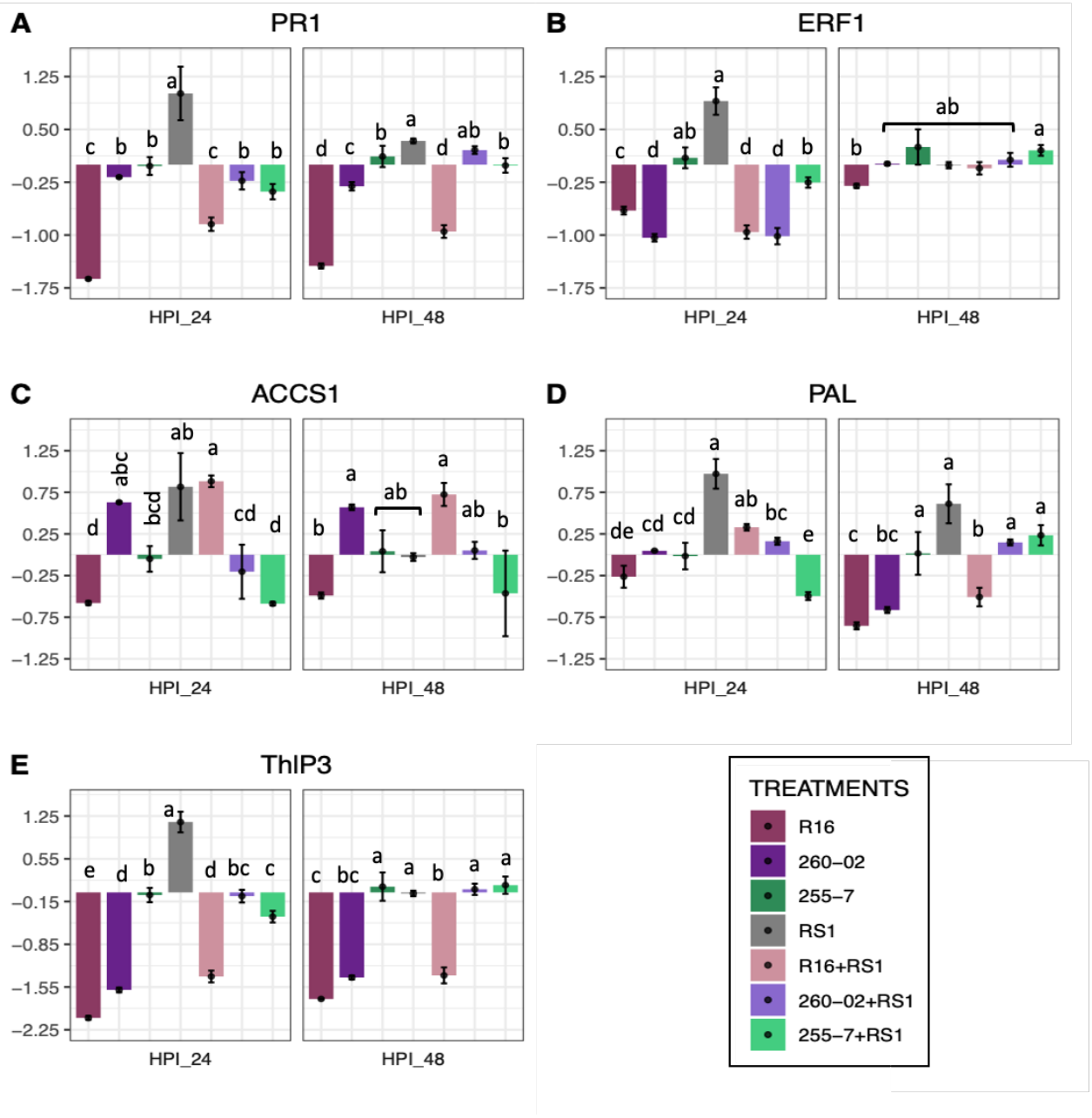

Figure 4. Barplots showing the effect of bacterial strains on the activity of defense related genes in the leaves of three weeks old Lattuga Romana seedlings challenged with/ without RS1 sampled at 24- and 48-hour post inoculation (hpi). *(A) Pathogenesis-related protein 1 (PR1), (B) Ethylene Response Factor1 (ERF1), (C) AminoCyclopropane-1-Carboxylate Synthase (ACCS1), (D) Phenylalanine Ammonia Lyase (PAL), and (E) Thaumatin-like Protein 3 (ThIP3). Y-axis represents $2^{-\Delta\Delta CT}$ value expressed as log10 of each gene, normalized on Adenosine Phosphoribosyl Transferase 1 (APT1) gene, and X-axis represents different treatments. Mean values display five replicates each treatment ± standard errors (SE). Different letters (a-e) on each bar indicate significant differences based on either Kruskal-Wallis non-parametric test (A, B, D, and E) followed by P-adjusted values using Benjamini and Hochberg (BH) methods or One-Way Anova (C) followed by multiple comparison Tukey HSD test (P < .05).*

4. Discussion

Exploitation of endophytic plant growth promoting bacteria (ePGPB) can be contemplated as a suitable and sustainable alternative to overcome the concerns regarding chemical fertilizer practices (Kandel et al., 2017). As, the competency of ePGPB to colonize within the host plants and to persist in the rhizosphere and soil, is an imperative trait to aid nutrient acquisition for growth promotion and biocontrol efficiency against various phytopathogens.

With this perspective, we performed three experiments (*In planta* and *In vivo*) in order to evaluate six endophytic bacteria (*Paenibacillus pasadenensis* R16 strain, *Pseudomonas syringae* 260-02 strain, *Bacillus amyloliquefaciens* strain CC2, *Lysinibacillus fusiformis* strain S4C11*, Paraburkholderia fungorum* strain R8 and *Pantoea agglomerans* strain 255-7) that have shown great potential to combat several pathogens that attack numerous crops (Passera, 2017; Passera et al., 2020, 2019). For that, soil-borne pathogen *Rhizoctonia solani* strain RS1 was studied as a test pathogen using four different lettuce varieties in order to determine a wide range biocontrol capability of selected bacterial strains.

In the seed germination assay, we found that three out of six strains (R16, 255-7 and S4C11) demonstrated the significant plant growth potential towards various *Lactuca sativa* L. varieties even in the presence of RS1 infection. For strain R16, this is in accordance with the previous results that demonstrated high percentage of seed germination in *R. solani* infected soil containing Romaine lettuce (Passera et al., 2020). The present study suggested that the strain R16 can promote growth and counteract the same pathogen in wide range of variety belonging to same host, particularly in *L. sativa acephala (*Lattuga Rosa*)* and *L. sativa capitata* (Lattuga resistente). Whereas, the strain 255-7 was able to perform preeminent role in all lettuce varieties, irrespective of pathogen presence. As per our knowledge, this is by far the first study demonstrating plant growth promoting and biocontrol potential of strain 255-7 against *R. solani* in *L. sativa acephala (*Lattuga Rosa*), L. sativa capitata (*Lattuga Meraviglia d'inverno*), L. sativa* L. var. *Longifoglia (*Lattuga Romana*)* and *L. sativa capitata (*Lattuga la Resistente*)*. The efficacy could be related to indole-3-acetic acid (IAA) production by this strain. As a similar study was conducted by Nabrdalik and colleagues (2018), that reported the involvement of indole-3-acetic acid (IAA) produced by endophytic *Pantoea agglomorans* strains BC17 and BC45 aiding in growth promotion and biocontrol against *R. solani* infected sugar beet. The third promising strain S4C11 showed better results only in *L. sativa* L. var.

Longifoglia (Lattuga Romana) infected with *R. solani*. This study is in agreement with the previous studies conducted by Passera (2017), in which strain S4C11 demonstrated antifungal potential against *Aspergillus nigri*, *Botrytis cinerea*, *Phomopsis viticola*, and *R. solani*, by inhibiting the growth of these fungi from 54% to 100% after an incubation of 14 days in tomatoes and lettuce. Furthermore, similar study of *Lysinibacillus fusiformis* demonstrated IAA production attributes (an auxin phytohormone) that is known for growth regulation in plant (Damodaran et al., 2019).

Proceeding with the second assay, our results revealed that the application of these three strains (R16, 260-02 and 2557) in the RS1 infected soil of romaine lettuce might have an effect on colonization with the rhizosphere as well as in the soil, indicating the probability of having both indirect (rhizosphere) and direct (soil) antagonistic feature against the pathogen. This finding is in relevance with the results obtained in studies conducted by Fatouros and colleagues (2018), as well as Aggeli and colleagues (2020). Both studies demonstrated the reduction in rhizosphere population with great biocontrol activity against *R. solani* and other pathogens which is mediated through antibiosis and some indirect mechanisms.

Given that the performance in the rhizosphere and soil depends on several traits, the negative correlation between both above-mentioned parameters have suggested that an indirect mechanism may be involved in rhizosphere soil, whereas, the observation of inoculation in the soil allowed us to notice a direct antifungal effect which may involve the processes of antibiosis or production of secondary metabolites such as VOCs. A recent study showed relevance, conducted by Raza and colleagues (2020), that VOCs derived from the three most common genera of ePGPB (*Bacillus*, *Paenibacillus* and *Pseudomonas*) was positively correlated with the pathogen suppression, however, the produced VOCs belonged to a diverse set of functioning.

The insights we obtained from the gene expression analysis marked the ability of our strains in inducing examined gene expression of SA (PR1, PAL and Thlp3), and JA/ ET (ERF1 and ACCS1) mediated pathways at an early stage of RS1 infection i.e., 24hpi and 48hpi. The protective activity of strains 255-7 was configured by an upregulation of PAL and ThlP3 gene at 48hpi, suggesting a delay in the activation of SA mediated resistance against *R. solani*. Whereas, the slightly different pattern was found in the strain 260-02 in which PAL gene was up-streamed instantly and remained similar at both 24 and 48hpi, indicating directly towards SA-mediation pathways. Nevertheless, the response of R16 strain was entirely diverse from

the other two strains, by upregulating both PAL and ACCS1 gene occurred as the co-activation of SA- and JA/ ET mediated resistance at 24hpi, suggesting an enhanced priming action of the strain. These results are in agreement with findings of Fatouros et al (2018), that showed the application of *Paenibacillus alvei* K165 strain against the necrotrophic fungi *Sclerotinia sclerotiorum* generates the simultaneous upregulation of PR1, ERF1 and Lipoxygenase (LOX) genes, markers of SA and JA/ET. Therefore, it is tempting to speculate that the ePGPB strain R16 triggers biocontrol efficacy against *R. solani* that seems to lie in the additive effect of co-activation of SA and ET/JA mediated defenses.

5. Concluding Remarks

The findings of the present study highlighted the plant growth promotion and biocontrol efficiency of strains R16, 260-02, 255-7 and S4C11 against soil-borne fungi *R. solani* at pre-harvest stage. The pathogen inhibition activity by strains R16, 260-02 and 255-7 in rhizosphere and soil can be attributed to antibiosis and ISR as direct and indirect antagonists, that leads to the activation of defense-related genes mediated by either SA pathway or in consortium of SA and ET/JA mediation. Additional research may be required to establish the current observations and open new insights on the use of these endophytes in the farming industry.

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3

ePGPBs biocontrol potential against Botrytis cinerea strain MG53 at post-harvest level and its impact on nutritional properties

The contents from this Chapter are published in Postharvest Biology and Technology

Cocetta, G., Passera, A., Vacchini, V., Shahzad, G., Cortellino, G., Picchi, V., Ferrante, A., Casati, P., Piazza, L., 2020. Postharvest Biology and Technology Use of microbial inoculants during cultivation maintain the physiological, nutritional and technological quality of fresh-cut romaine lettuce. Postharvest Biol. Technol. 111411.
https://doi.org/10.1016/j.postharvbio.2020.111411

Abstract

Nutrition-sensitive agriculture is a novel concept in the agri-food system, which considers the implementation of techniques able to guarantee the nutritional value of the produce, the sustainability of the production and, at the same time, to reduce the ecological impact of agricultural practices. These principles can be also introduced in the fresh-cut market with the aim of maintaining the produce quality during shelf life. In this context, the use of bio-based products is rapidly increasing for improving economic and environmental sustainability of cropping systems during cultivation and shelf life. The aim of this work was to evaluate the effects of three different endophytic plant growth promoting bacteria (ePGPB)-based formulations (*Paenibacillus pasadenensis*, *Bacillus amyloliquefaciens*, *Pseudomonas syringae*) applied during romaine lettuce cultivation by monitoring the changes of nutritional quality indexes at harvest and during storage. Results showed that the application of microbial inoculants during romaine lettuce cultivation contributed to the maintenance of nutritional quality attributes of leaves during shelf life. The microbial inoculants prevented the development of postharvest fungal pathogen *B. cinerea*. Moreover, the study evidenced different modes of action of the different inoculants, and, in the case of *Pseudomonas syringae* strain 260-02 application, a direct involvement of ascorbic acid-mediated antioxidant mechanisms was observed.

Keywords*: Antioxidants, Bio-based products, endophytic plant growth promoting bacteria (ePGPB)*, *Botrytis cinerea, Leafy vegetables, Nutrition-sensitive agriculture, Postharvest quality*

1. Introduction

The fresh-cut market has been constantly increasing in importance and economic relevance in the recent years. Within this sector, salads are among the most important vegetables used and appreciated by the consumers. The growing demand for fresh and high quality minimally processed products and the awareness of consumers towards the rise of environmental issues, are pushing toward the adoption of novel agronomical and technological practices aiming to preserve both the product quality and the environment sustainability (Shabbir et al., 2019). The list of banned pesticides is constantly being updated, including more and more molecules, and this phenomenon is driving the research effort towards finding new effective and reliable alternatives. In this context, the adoption of nutrition-sensitive agriculture (NSA) is of particular importance. NSA is a novel concept in the agri-food system, which considers the implementation of techniques able to guarantee the nutritional value of the produce, the sustainability of the production and, at the same time, to reduce the environmental impact of agricultural practices (Shetty, 2018). Fresh-cut salads production pipeline is characterized by a sequence of mild operations (including washing, cutting, drying, packaging, and storage) which, on a physiological point of view, represent a stress for the leaf tissue. The loss of quality during postharvest can be due to enzymatic phenomena and/or by the proliferation of saprophytic and pathogenic bacteria or fungi, which rapidly lead to the total loss of product marketability (Lugtenberg et al., 2017).

This is particularly true when speaking of a major post-harvest pathogen for lettuce: *Botrytis cinerea*. While it is known that this pathogen can cause crown and bottom rot in salads pre-harvest (Sowley et al., 2010, Chatzidimopoulos and Pappas, 2019; Sanogo et al., 2019), this necrotrophic pathogen is most devastating for the grey mold it causes on leaves in post-harvest conditions (Van Kan, 2005; Shim et al., 2013; Barrière et al., 2014).

The use of bio-based products in agriculture is rapidly growing in order to increase the economic and environmental sustainability of cropping systems. Biocontrol agents are characterized by a low environmental impact, especially in comparison to synthetic fertilizers. Scientific efforts have been dedicated in the last years in the identification of the mode and mechanism of action of various bio-based products to be used in agriculture. It has been demonstrated that the application of bio-based products could improve the agronomical performance of crops, and at the same time it could also enhance the nutritional value of produce, by stimulating the biosynthesis and the accumulation of bioactive molecules (such as phenolic compounds) and vitamins (such as ascorbic acid, or carotenoids) (Cocetta and

Ferrante, 2020). Also, there is no clue regarding the effect on postharvest quality deriving from the field application of potential biocontrol agents.

For this reason, the aim of this work was to evaluate the effects of three different endophytic plant growth promoting bacteria (ePGPB)-based biostimulant formulations applied during romaine lettuce cultivation by monitoring the changes of several nutritional indexes at harvest and during storage. These ePGPBs (*Paenibacillus pasadenensis* strain R16, *Bacillus amyloliquefaciens* strain CC2, and *Pseudomonas syringae* strain 260-02) have been previously used as biocontrol agents against soilborne pathogens of lettuce *Rhizoctonia solani* and *Pythium ultimum*, showing a reduced severity of the symptoms induced by these pathogens on treated lettuce plants, while having no negative effects on the quality of the produce at time of harvest or altering the microbial diversity of bulk soil (Passera et al., 2020). The hypothesis of the work was that these three ePGPBs could increase the product quality with positive effects also during the shelf life. Lettuce has been chosen as the most representative and widely used crop in the fresh-cut salads industry (Tudela and Gil, 2020). The parameters considered for the experimental evaluations included those related to the nutritional value (phenolic compounds, carotenoids, vitamin C and antioxidant capacity). Moreover, the potential biocontrol effect of inoculants was assessed by measuring the damage caused by a typical postharvest fungal pathogen (*Botrytis cinerea*).

2. Material and Methods

2.1. Microbial strains

Three ePGPB strains were used as candidate biocontrol and postharvest quality-promoting agent. The applied ePGPB strains were: *Paenibacillus pasadenensis* strain R16, *Pseudomonas syringae* strain 260-02, and *Bacillus amyloliquefaciens* strain CC2.

Strain R16 has been isolated from grapevine leaf in 2009 (Bulgari et al., 2011), and has been characterized as an antifungal agent *in vitro* against *Botrytis cinerea* and *Phomopsis viticola* (Passera et al., 2017), and *in vivo* against *B. cinerea*, *Rhizoctonia solani*, and *Pythium ultimum* (Passera et al., 2020). Based on the obtained results and the characterization of the genome (Passera et al., 2018), the main modes of action of this strain seem to be the production of chitinase, antifungal volatile organic compounds, and indirect effects that strengthen plant defenses.

Strain 260-02 has been isolated from roots of apple trees in 2012 and has been characterized as an *in vitro* antifungal agent against *B. cinerea*, and *in vivo* against *B. cinerea*, *R. solani*, and *P. ultimum* (Passera et al., 2019; Passera et al., 2020). Based on the previously obtained results and the characterization of the genome, the main modes of action of this strain seem to be the production of toxins, siderophore, and antifungal compounds, as well as the activation of plant defense mechanisms, as suggested by effective biocontrol against also against a viral pathogen (Passera et al., 2020).

Strain CC2 has been characterized as a potential *in vivo* antifungal agent against *R. solani* and *P. ultimum* (Passera et al., 2020).

All these strains were successfully applied during romaine lettuce cultivation and their effectiveness in containing the damage caused by soilborne fungal pathogens has been demonstrated (Passera et al., 2020). All strains were cultivated on LB High Salt Agar plates (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, sodium chloride 10 g L^{-1}, agar 15 g L^{-1}) at 25 °C and were stored in a 20 % glycerol solution at -80 °C for long conservation periods.

2.2 Plant material and Inoculum with bacterial strains

Romaine lettuce (*Lactuca sativa* L. var. *longifolia*) seedlings were grown in 13 cm pots filled with commercial potting soil. Two weeks old seedlings were inoculated with the ePGPB strains, which were administered as soil drenching. The treatments were: R16, 260-02, or CC2 (10^5 CFU mL^{-1} in Ringer's solution), while sterile Ringer's solution was used as non-treated

control (NT). Seven plants were used for each treatment. The plants were grown in an experimental greenhouse under monitored conditions (25 ± 3 °C, 14 h photoperiod) and were harvested after three weeks from transplant.

2.3. Botrytis cinerea inoculation

Additional lettuce plants, 10 per treatment, were prepared as detailed in section 2.2. These plants were used for inoculation of the postharvest foliar pathogen *Botrytis cinerea*, strain MG53 (which will be identified as BC for the rest of the study). The inoculum of BC was composed by a conidia suspension (10^5 conidia mL^{-1}) obtained by adding sterile water and scraping the surface of well-developed BC mycelium, grown on PDA medium for ten days. The suspension was filtered on double-layer sterile gauze to remove the mycelium fragments. The concentration and purity of the conidia suspension was assessed by visual analysis in optical microscopy (20X; Easylab CX40, Olimpus), evaluating five 10 µL drops per 5 mL batch of suspension in a Kova counting grid, and then diluted to the final concentration of 10^5 conidia mL^{-1}. The inoculum of BC conidia suspension was carried out five days after the inoculum with the ePGPB strains and consisted of spraying 15 mL of conidia suspension on each plant, ensuring a homogeneous distribution of the droplets on the leaves. These BC-inoculated plants were kept in a different greenhouse from the non-BC-inoculated plants, albeit with the same conditions. The non-BC-inoculated plants were used as healthy control plants to compare the development of symptoms.

2.4. Evaluation of effectiveness of microbial strains in controlling development of Botrytis cinerea

The development of symptoms was monitored during growth in greenhouse and in shelf-life conditions, obtained by keeping the leaf material in high humidity conditions (95 %) and 24 °C of temperature for ten days. The leaf material collected at harvest was divided into two separate trials: i) excising from the leaves disks with a diameter of 2 cm, making three biological replicates of 10 disks each per treatment, stored on 1 % agar-water plates and ii) storing a whole leaf per plant per treatment in a humid chamber. For both types of samples, the symptoms were visually assessed and assigned to a symptom severity class ranging from 0 (asymptomatic material) to 7 (material showing 100 % of BC infection and sporulation) as previously reported (Vercesi et al., 2013). The symptom classes were then converted to an

infection percentage index (I%I) using the formula presented by Townsend and Heuberger in 1953.

2.5. Evaluation of nutritional properties

2.5.1. Ascorbic and dehydroascorbic acid

For each treatment/time, three replicates of 2 g of leaf tissue were ground in pre-chilled mortar with liquid nitrogen and the powder was immediately added to 5 mL of 3 % metaphosphoric acid. The homogenate was then centrifuged at 25,000 x g for 15 min at 4 °C, and the supernatant immediately analyzed. L-ascorbic acid (AsA) was quantified by HPLC as previously described (Picchi et al., 2012). The oxidized form (dehydroascorbic acid, DHA) was determined by the "subtractive" method after measurement of the total ascorbate (AsA + DHA) content following reduction with 100 mM Tris-carboxyethyl phosphine (TCEP) in 0.1 M HCl. The reduction was carried out according to Wechtersbach and Cigić (2007). Reduced extracts were then diluted with 0.02 M orthophosphoric acid and immediately analyzed by HPLC. The analytical column was a 250 x 6 mm i.d., Inertsil ODS-3, maintained at 40 °C. The isocratic elution was performed using 0.02 M mobile phase orthophosphoric acid at a flow rate of 0.7 mL min^{-1}. Samples of 20 µl were injected and monitored at 254 nm. The identity of the AsA peak was confirmed by coelution with authentic standards and the concentration of AsA was calculated from the experimental peak area by analytical interpolation in a standard calibration curve (range 0.0025-0.02 g L^{-1} AsA).

2.5.2. Phenolic index and total anthocyanins

Phenolic compounds were extracted from leaves disks (around 50 mg) that were placed in 5 mL of acidified methanol (1 % HCl v/v) and maintained at 4 °C for 24 hours in the dark. The total phenolics content was expressed as phenolic index, calculated as the absorbance measured at 320 nm (Ke and Saltveit, 1989). Total anthocyanins were assayed from the same extracts by spectrophotometric readings at 535 nm and the concentration expressed as cyanidin-3-glucoside equivalents (g $kg^{-}1$) was calculated using the extinction coefficient (ε) of 29,600 mM^{-1} cm^{-1} (Klein and Hagen, 1961).

2.5.3. Total carotenoids

For total carotenoids, leaf discs (around 40 mg) were placed in pure methanol (99.9 %) and extracted overnight at 4 °C in the dark. Leaf samples then were assessed by measuring the

spectrophotometrical readings at 470 nm and carotenoid concentrations were calculated using Lichtenthaler's formula (Lichtenthaler, 1987).

2.5.4. Antioxidant capacity (DPPH)

The antioxidant capacity was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical quenching method. For each treatment/time, three replicates of 2 g of leaf tissue were ground in pre-chilled mortar with liquid nitrogen and the powder was immediately added to 5 mL of 1:1 v/v mixture of ethanol and 0.06 N HCl. The homogenate was then centrifuged at 25,000 x g for 15 min at 4 °C, and the supernatant was used as extract. The DPPH quenching capacity was measured using electronic paramagnetic resonance (EPR) with a MiniScope MS200 Magnettech (Berlin, Germany) following the protocol detailed in Picchi et al. (2012). Data are presented as ascorbic acid equivalents (g kg^{-1}) on fresh weight basis.

2.6. Statistical analysis

All data were subjected to the analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. Statistics were performed using GraphPad Prism version 6 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

3. Results

3.1. Evaluation of effectiveness of microbial strains in controlling development of Botrytis cinerea

No visual symptoms caused by *Botrytis cinerea* (BC) were detected during crop cultivation. During the post-harvest shelf-life monitoring, in both leaf disk and whole leaf condition, no symptoms were detected in non-BC-inoculated leaves. In contrast, leaves from plants that were inoculated with BC showed varying grade of rotting and molding, compatible with infection from BC. The results of this evaluation are reported in Figure 1.

The non-treated plants inoculated with BC showed high levels of infection in both assays, having an average value of infection percentage index (I%I) of 44 % and 90 % in leaf disk and whole leaf assay, respectively. Plants that were treated with the bacterial inoculants all showed statistically significantly lower I%I values in both assays: CC2 had average I%I of 6 % and 42 %, 260-02 had average I%I of 12 % and 33 %, and R16 had average I%I of 11 % and 9 %, for leaf disk and whole leaf assay respectively.

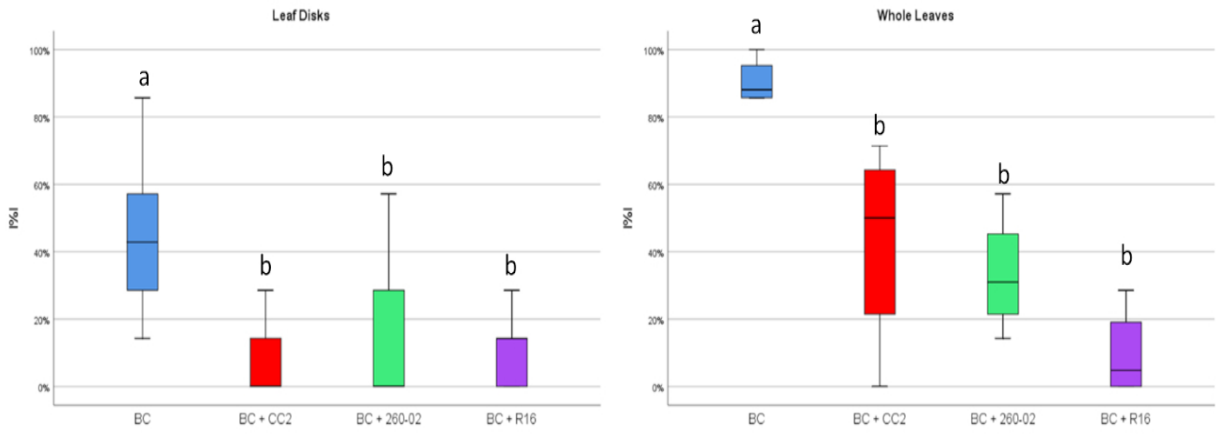

Figure 1. Results of B. cinerea inoculation trials. *The graphs report on the X-axis the different treatments (plant inoculated with BC conidia and either non-treated [NT + BC], or treated with strains CC2 [CC2 + BC], 260-02 [260-02 + BC], and R16 [R16 + BC]), while the Y-axis reports the infection % index (I%I). Data are means ± SE. Different letters (a, b) indicate statistically significant differences in the results, according to a One-Way ANOVA followed by Bonferroni post-hoc test ($P < 0.05$).*

3.2. Evaluation of nutritional properties of fresh-cut lettuce

Ascorbic acid (AsA) (Fig. 2 A) and total vitamin C (AsA + DHA) (Fig. 2 B) showed a significant decline during storage (statistics not shown) and in general, treatments allowed maintaining higher levels of both AsA and DHA. The positive effect of treatments was significant in case of AsA at harvest and after one day of storage. The maximum AsA content (0.081 g kg^{-1}) was registered in leaves from 260-02 treated plants at harvest. The highest amount of AsA+DHA was recorded in leaves from R16-treated plants at the end of shelf-life. No changes were found in the phenolic index, total anthocyanins, and total carotenoids (Fig. 2 C, D, E).

A marked increment in the antioxidant capacity was recorded at harvest, as a response to the application of 260-02 (Fig. 2 F), and after 1 day of shelf-life, 260-02-treated lettuce maintained significantly higher DPPH quenching capacity.

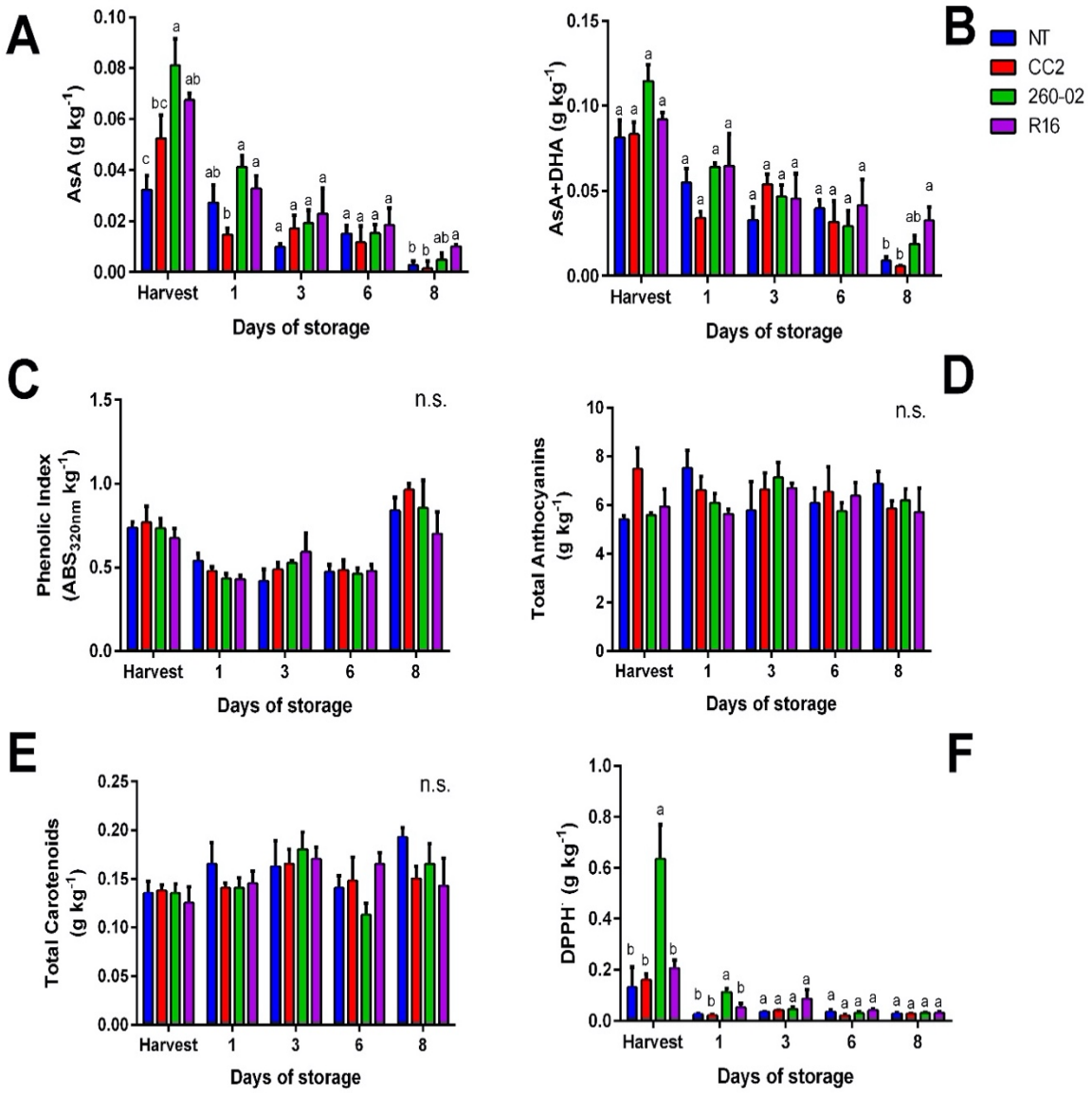

Figure 2. Nutritional properties of romaine lettuce leaves at harvest and during storage, as affected by different treatments. *A: Ascorbic acid; B: Total vitamin C; C: Phenolic index; D: Total anthocyanins; E: Total carotenoids; F: Antioxidant capacity. Data are means ± SE. At each time point, different letters indicate statistically significant differences among treatments, according to a One-Way ANOVA followed by Bonferroni post-hoc test ($P < 0.05$).*

4. Discussion

The damage caused by *B. cinerea* on salads is so relevant that solutions for this problem has been sought for a very long time (Wood, 1951). The most direct and obvious solution to the problem is the use of fungicides, but this strategy has several limitations: the most evident is the economic impact of these treatments, that can often cost more than the damage caused by the pathogen (Fortunati et al., 2017); the risk of selecting resistant strains of the pathogen by repeated applications of the same fungicide is also a concern (Spotts et al., 1986); moreover, the environmental impact of the treatment with synthetic fungicides (Komarek et al., 2010), is an aspect that must be considered, especially under the guidelines of NSA that advocate a more sustainable production. For these reasons, several alternatives to the use of synthetic fungicides have been researched, including both postharvest treatments with molecules with a lower environmental impact, such as chitosan (Fortunati et al., 2017), and preharvest treatments of different nature, including organic compounds (Zlotek and Wojcik, 2014), UV light treatments (Vasquez et al., 2017), and biocontrol agents (De Meyer et al., 1998). The pre-harvest approaches in particular offer several benefits as they do not rely on the application of active substances on the edible part of the plant, but rather reinforce the plant defense systems which, in addition to providing resistance to the pathogen, can improve the quantity of desirable molecules (such as antioxidants) in the produce increasing its nutritional value, as expected for the nutrition sensitive agriculture (NSA) proposition.

All three endophytic plant growth promoting bacteria (ePGPB) strains tested in this study provided a noticeable amount of protection against *B. cinerea* to the treated lettuce plants through an indirect biocontrol mechanism, as evidenced by the fact that reduced symptoms were observed without a direct contact between the beneficial microorganisms (inoculated in the soil) and the pathogen (inoculated directly on the leaves). The indirect nature of this biocontrol effect is further reinforced when taking into consideration the results previously obtained with these same strains in the control of soilborne pathogens: microbiota analysis of rhizosphere, roots, and bulk soil revealed that the inoculated strains were no longer detectable in any of these compartments three weeks after inoculation, but operated a restructuring of the plant-associated microbiota (rhizosphere and root) while leaving the bulk soil largely unaltered (Passera et al., 2020). Considering these previous results that suggest a very little environmental impact of the inoculation with these strains, and the previously obtained results on soilborne pathogens, the selected bacteria show very promising results for a future application, in particular *P. pasadenensis* strain R16.

These observations have been corroborated by the evaluation of the nutritional status of the leaves. In fact, lettuce treated with 260-02, showed a significant increment in AsA content and improved *in vitro* antioxidant capacity, in correspondence to the abovementioned increment in stress responses. Considering that this *Pseudomonas syringae* strain 260-02 has been already proven to be an effective biocontrol agent (Passera et al. 2019; Passera et al., 2020), it is possible to hypothesize that it would have stimulated the plant defense mechanisms involving the production of reactive oxygen species (ROS). In this case we observed a prompt response of lettuce leaves, which immediately activated a series of defense mechanisms, which would had probably involved the AsA metabolism instead of other antioxidants. The predominant role of AsA is further supported by the lack of changes in the phenolic compounds, anthocyanins and carotenoids content. Among antioxidant molecules, AsA has been reported to be one of the most sensitives to stressful conditions and it is often reported as a good marker of oxidative responses in fresh-cut leafy vegetables (Ferrante et al., 2009). Moreover, a recent study suggests an important role for AsA in controlling H_2O_2 accumulation during the application of a plant growth-promoting bacteria in rice (Kumar et al., 2019), so it is possible that AsA could play a similar role in case of romaine lettuce. Concerning the other treatments, it is interesting to notice, that at the end of storage, the lower oxidative stress incidence and the maximum vitamin C content was recorded in leaves from R16-treated plants. This is particularly interesting considering the promising results obtained by this treatment in the control of *B. cinerea* and suggest the possibility of efficiently control the pathogen development by stimulating the plant own defense mechanisms, with no negative drawback on nutritional properties.

5. Concluding Remarks

Based on the results obtained it is possible to conclude that the application of microbial inoculants during romaine lettuce cultivation could contribute to the maintenance of nutritional quality attributes of leaves during shelf life. At the same time, the microbial inoculants were proved to be useful in preventing the development of postharvest fungal pathogen *B. cinerea.* Moreover, the study helped in individuating potentially different modes of action of the different inoculants, and, in the case of *Pseudomonas syringae* 260-02, it can be hypothesized a direct involvement of AsA-mediated antioxidant mechanisms.

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SUMMARIZING DISCUSSION

Summarizing Discussion

The plant immune system is primarily composed of immune receptors that detect pathogen-derived signatures, particularly, the disease resistance (R) receptors, which detect pathogen virulence effectors attempting to subvert host defenses. These receptor (R) proteins are present across the green plant lineage and typically encode nucleotide-binding leucine-rich repeat (NLR) intracellularly that targets effector proteins directly or indirectly through hormone signaling pathways regulated by Jasmonic acid (JA) or Ethylene (Et) and Salicylic acid (SA) mediators (Backer et al., 2019). Upon the compromised host plant immune system, the pathogen successfully invades the entire plant that ultimately leads to consequent destruction.

Plant diseases pose humongous biotic stress and limits the production of agriculturally important crops, accounting for 31-42% yield loss worldwide (Moustafa-Farag et al., 2020). As a matter of fact, the world's population is growing and so does the food demand. High yield production of crop varieties that require little to no synthetic pesticides or fertilizers are fundamental to sustainably producing sufficient food to satisfy this rising demand (Sayyed, 2019). As this awareness has been reaching around the globe, it is a prerequisite for alternative and more sustainable ways to practice modern agriculture. From the past decades, several biological control strategies has been practiced against phytopathogens which are considered as a great contribution towards sustainable agriculture (Van Driesche et al., 2010). Speaking of the terminology of biocontrol in plant pathology, it aims to reduce the population of target organisms by utilizing the potential beneficial microbes (antagonists of pest or pathogen), or its molecules to suppress phytopathogens' ability to colonize the host or induce symptoms that causes the plant diseases (Heimpel and Mills, 2017).

In this context, the general scope of the present doctoral studies was centered on exploiting two promising biocontrol strategies i.e., (i) the use of endophytic plant growth promoting bacteria (ePGPBs) as microbial inoculants, that offers not only biocontrol effects against pathogens but can also promote plant growth via endosymbiotic relationships, through several mechanisms of actions; and (ii) exogenously applied RNA interference (RNAi) based product derived from double-stranded RNA (dsRNA) molecules, that offers the promise of a reduced environmental impact to pests/ pathogens control.

Regarding the first strategy, though the isolation of potential beneficial microbes can be challenging since there are no specific indicators to determine whether a microbe is capable of having a beneficial effect on a host plant without assessing the microorganism's effect. Decades of research have provided few guidelines in the literature, such as, choosing microbes isolated from plant tissues or soil and signifying their ability to live in the right ecosystem since some genera are better known for their plant-growth promoting and/or biocontrol ability (Passera, 2017). Furthermore, many studies have been conducted to understand and differentiate beneficial microbes' mode of actions and its effect on the host plant such as: antibiosis, competition for nutrients and space, plant growth promotion, and mediation of induced systemic resistance. Scientists are now seeking in-depth knowledge to describe the alterations of microbial community present in the diseased host plant with the healthy ones that can possibly lead to the restructure of microbiota, preventing the pathogen attack or inducing recovery from diseases while causing least to no ecological impact outside the host plant and keeping up the nutritional status (Bulgari et al., 2011; Cocetta et al., 2020; Larousse and Galiana, 2017; Passera et al., 2020; Podolich et al., 2015).

Since the past decades, ePGPBs have been intensively exploited in the biological market against variety of phytopathogens, and comprises mostly in the genera of *Pseudomonas, Microbacterium, Pantoea, Bacillus, Micrococcus, Stenotrophomonas,* and *Burkholderia* (Aswani et al., 2020; Shi et al., 2014; Sun et al., 2008). Based on the previous promising findings, the following five ePGPB strains (*Paenibacillus pasadenensis* R16 strain, *Pseudomonas syringae* 260-02 strain, *Lysinibacillus fusiformis* strain S4C11, *Paraburkholderia fungorum* strain R8, and *Pantoea agglomerans* 255-7 strain) were found to be characterized for their plant growth promotion and biocontrol competency through several assays carried out at different levels (*in vitro*, *in vivo*, *in planta*) against various phytopathogens (mainly viruses, fungi and bacteria) on numerous model plants and host plants Passera (2017). Hence, the present doctoral studies were conducted to supplement those previous findings to elucidate in-depth knowledge behind this plant growth promotion (PGP) and biocontrol competencies that might have direct, indirect or simultaneous effects on two target pathosystems, being categorized as Aim 1 (viruses that are infecting Solanaceae family) and Aim 2 (fungal (pre-/ post-harvest) pathogens infecting Asteraceae family).

The chapter 1 of Aim 1 was corresponded to test all five ePGPB strains *in planta* against four viruses (*Cymbidium ringspot virus* (CymRSV), *Cucumber mosaic virus* (CMV), *Potato*

virus X (PVX), and *Potato virus Y* (PVY)) on *N. benthamiana* plants and were compared with three chitosan-based products that are known to induce resistance in plants. The objectives of this study were to evaluate their role in providing plant PGP, broad-spectrum protection, the effect and relevance of respective virus concentration on the symptom's severity, and analysis of defense related genes (*Enhanced Disease Susceptibility-1* (EDS1), *Non-expressor of Pathogenesis-related genes-1* (NPR1), and *Pathogenesis-related protein-2B* (PR2B)) to understand a possible mechanism of action behind the biocontrol effect that was registered towards the respective virus.

The chapter 1 of Aim 2 was corresponded to test three ePGPB strains (R16 strain, 260-02 strain, and commercial product *Bacillus amyloliquefaciens* (CC2 strain)) against *Rhizoctonia solani* and *Pythium ultimum* infected *Lactuca. sativa* L. var. *longifoglia* (Romaine lettuce) at pre-harvest stage and were compared with the commercially available *Trichoderma* spp.-based product. The objectives were to evaluate PGP, biocontrol potential, and then their ecological impact on non-infected phytomicrobiome (sterile and non-sterile roots, rhizosphere and soil).

The results of chapter 1 of Aim 2 provided the baseline for further study, as indicated in chapter 2, in which these ePGPB strains (R16, 260-02, and CC2) along with the other stains S4C11, R8, and 255-7 were tested for their putative broad-spectrum abilities. The viable seed germination assays were conducted by using four different varieties of lettuce (*L. sativa acephala* (Lattuga Rosa), *L. sativa capitata* (Lattuga Meraviglia d'inverno), *L. sativa* L. var. *longifoglia* (Lattuga Romana) and *L. sativa capitata* (Lattuga la Resistente)). Whereas, serial dilution and plating methods, and analysis of defense related genes (*Pathogenesis-related protein 1* (PR1), *Phenylalanine Ammonia Lyase* (PAL), *Thaumatin-like Protein 3* (ThlP3), *Ethylene Response Factor1* (ERF1), and *1-AminoCyclopropane-1-Carboxylate Synthase* (ACCS1)) were evaluated by using Romaine lettuce only.

Furthermore, the study of chapter 3 was conducted in relevance with chapter 1, in which the biocontrol potential of ePGPB strains (R16, 260-02, and CC2) were tested against *Botrytis cinerea* strain MG53 infection in *L. sativa* L. var. *longifoglia* (Romaine lettuce) at post-harvest level and how they may impact the nutritional properties (phenolic compounds, total anthocyanins, total carotenoids, vitamin C and antioxidant capacity measure) during cultivation and shelf life.

Here, some of the peculiar characteristics of each ePGPB strain are concluded based on the present study with the relevance of past studies.

Paenibacillus pasadenensis R16 strain was previously found to possess several plant-growth promoting and antifungal traits, demonstrated through *in vitro* trials, and also to exert biocontrol on different pathogens (*P. syringae*, *B. cinerea*, and CymRSV) during *in vivo* and *in planta* studies. These findings are discussed in the studies conducted by Passera (2017). In the present study, strain R16 have shown great potential in promoting growth of plants of *N. benthamiana* (healthy and CymRSV, CMV and PVX infected plants) and *Lactuca sativa* L. (healthy plants of *L. sativa capitata*, as well as *R. solani* infected *L. sativa* L. var. *longifoglia,* and *L. sativa acephala* plants) as indicated in Chapter 1 of Aim 1, and Chapter 2 of Aim 2, respectively. The biocontrol capabilities were shown with significant reduction in symptoms severity caused by viruses (CymRSV and CMV infected *N. benthamiana*) and pre-harvest, soilborne fungi (*P. ultimum* and *R. solani* infected *L. sativa* L. var. *longifoglia*) as well as post-harvest fungal pathogen *B. cinerea*. In addition to that, microbiota analysis of romaine lettuce rhizosphere, roots, and bulk soil revealed no detection of R16 strain in any of these compartments three weeks after inoculation but operated a restructuring of the plant-associated microbiota (rhizosphere and root) while leaving the bulk soil largely unaltered (Passera et al., 2020), suggesting that this strain, while effective in controlling the soilborne diseases, does not exert a fierce competition with the native microbiota: it stimulates the beneficial bacteria naturally present in the rhizosphere and root, and does not largely affect soil microbiota. Furthermore, the application of strain R16 revealed possible mechanism of interaction via direct means through reduction in rhizosphere and soil population of *R. solani* infected romaine lettuce (antibiosis, confirming the involvement of key molecules of terpenes i.e., DMNT and farnesol) and indirect means through upregulation of EDS1 expression in *N. benthamiana* (SA-dependent ISR pathways) and upregulating both PAL and ACCS1 gene in *R. solani* infected *L. sativa* L. var. *longifoglia* (co-activation of SA- and JA/ ET mediated ISR resistance).

Pseudomonas syringae 260-02 strain was previously found to possess several plant-growth promoting and biocontrol, demonstrated through *in vitro* trials, to promote plant growth in several horticultural plants, and also to exert biocontrol on *P. syringae* strain DC3000 and *B. cinerea* during *in vivo* and *in planta* studies (Passera, 2017; Passera et al., 2019). The present study demonstrated that 260-02 was not only able to reduce symptoms but also promoted the growth of *N. benthamiana* in the presence of CymRSV, CMV, and PVX. Strain 260-02 showed

direct influence by reducing *R. solani* population, confirming the previous studies that suggested a broad-range biocontrol and plant-growth promoting ability of this strain. It is interesting to note that indirect interaction of the strain 260-02 triggered variations in different crops indicating SA mediated resistance pathways, such as upregulation of EDS1 gene in treated *N. benthamiana* plants, upregulation of PAL gene in *R. solani* infected romaine lettuce, as well as, AsA-mediated antioxidant mechanisms by showing higher levels of ascorbic acid production in *B. cinerea* infected romaine lettuce.

Paraburkholderia fungorum R8 strain was previously found to possess a vast number of plant-growth promoting traits, demonstrated through *in vitro* trials, but was less tested than the other strains regarding the effective biocontrol ability of the strain, having positive results only for in vivo assays carried out against *B. cinerea* on tomato and *P. syringae* strain DC3000 on pepper (Passera, 2017). The present study indicated that the strain R8 mediated the growth promotion and biocontrol efficacy only against CymRSV infected plants and no other viruses. This virus is also the one that, overall, gave lower intensity of symptoms, suggesting that strain R8 is not effective against viruses that can give severe symptoms. Considering also that this strain did not trigger any response in the investigated genes, it can by hypothesized that the benefit to plant health registered in the infection with CymRSV is not due to priming of plant defenses, but merely to plant growth promotion. It is also possible that the defense pathways involved in the interaction between strain R8 and the host plant are different from those mediated by SA in *N. benthamiana* plants.

Lysinibacillus fusiformis strain S4C11, showed similar results as strain R8 in biochemical and plant growth promotion, but its biocontrol efficacy was shown against *B. cinerea*, *P. viticola*, and *R. solani* (Tomato, Lettuce), and *P. syringae* pv. *tomato* strain DC3000 (Pepper); this strain is also the one that gave best results regarding plant-growth promotion in previous studies (Passera, 2017). The present study confirmed the results of previous study by accompanying higher seed germination rate in *R. solani* infected romaine lettuce. Furthermore, S4C11 strain stimulated plant growth promotion and biocontrol trait in CymRSV and CMV infected *N. benthamiana* plants. These results may be explained by the downregulation of PR2B transcripts, that was not accompanied by a change in expression levels in NPR1 and EDS1 genes, indicating an involvement of SA-independent defense pathways.

Pantoea agglomerans 255-7 strain, was shown to produce IAA, but did not produce siderophore and chitinase among the biochemical plant-growth promotion-associated traits that were examined. It promoted growth in pepper plants and reduced the pathogenicity of *B. cinerea* strain MG53 in tomato, *P. syringae* pv. *tomato* strain DC3000 and CymRSV in pepper plants (Passera, 2017). In the present study, strain 255-7 enhanced not only the growth of *N. benthamiana* in the presence of CymRSV, CMV and PVX but also showed higher seed germination rate in all four investigated lettuce variety in the presence of *R. solani*. Furthermore, this strain managed to reduce significantly the symptoms induced by CymRSV and CMV in *N. benthamiana,* and the *R. solani* population in rhizosphere and soil, indicating direct effect mediated by antibiosis. After treatment with this strain, *N. benthamiana* plants showed no effect on the expression of NPR1, PR2B and EDS1 genes, suggesting the involvement of SA independent pathways. In lettuce challenged with *R. solani*, treatment with strain 255-7 triggered PAL and ThlP3 gene upregulation indicating the induction of SA mediated pathways. These findings suggest that the strain 255-7 can acts indirectly via both SA and ET/JA mediating resistance, depending on its specific interaction with the host plant.

The difference in results that were obtained in the biocontrol assays that involved different bacteria (ePGPBs), pathogenic viruses and fungi, and host plants testifies once again how important each member in these multi-trophic interactions is.

The potential effectiveness of the study employed against viral pathogens to analyze how these ePGPB strains would interact with a respective virus against which direct biocontrol mechanisms are ineffective, allowing to investigate indirect mechanisms that might be at work. The technique was rather significant against CymRSV and CMV that showed an effect in inducing systemic resistance and priming of the host plant but not against PVX and PVY. Also, the study of relative gene expression reveals correspondences in the impact that ePGPB strains have on the host plant, strengthening the hypothesis that, despite the relative results obtained, the mechanisms underlying the viral pathogen's biocontrol are not the same.

Regarding the fungal pathogens at both pre- and post-harvest stage, application of ePGPB strains (particularly strains R16, 260-02, and 255-7) as microbial product proved their efficacy by showing promising results against *R.solani* which confirmed the previous findings (three level of interactions: (i) molecular cross-talk, (ii) colonization of the host, and (iii) alteration of host's gene expression) and strengthened our hypothesis of the present doctoral

study. Most importantly, the PGP in the presence and absence of pathogen (*R. solani*), counteracting the pathogen directly (both *P. ultimum* and *R. solani*) or/and indirectly (*R. solani*), boosting the microbial diversity of beneficial bacterial community shift in the rhizosphere and roots (e.g., *Oxalobacteraceae*, and *Burkholderiaceae*) with least or no ecological impact outside the host plant. And above all, by maintaining not only the nutritional quality attributes at the time of harvest but also the prevention of onset of postharvest fungal pathogen (*B. cinerea*) during the storage, which subsequently will increase the shelf-life and marketability of prolonged fresh produce. Such effect obtained by the ePGPBs have given a new insight of utilizing ePGPB based products in Nutrition-sensitive agriculture.

Furthermore, the mechanism of action of the strain 260-02 (*Pseudomonas syringae*) were brought into light by indicating direct involvement of ascorbic acid-mediated antioxidant mechanisms against *B. cinerea* and indirect involvement of SA mediated pathways against *R. solani*. In addition, based on the different verification and validation steps carried out by Passera (2019), proved to be a beneficial microorganism with a biological control potential. Hence, the present study fortified the foundation results and guaranteed that the strain 260-02 is not a pathogenic but a beneficial endophytic plant growth promoting bacteria.

The main challenges and limitations emerged during the assessment of the efficacy of the different ePGPB strains in the present doctoral study were as following: (a) the large number of variables and interactions that are involved in these pathosystems. For instance, the present study design did not acquire entire knowledge of host-pathogen-ePGPB interactions while investigating viruses, as well as, the gaps where phytomicrobiome study really lacked the knowledge of plants infected with *R.solani* and treated with ePGPBs; (b) the precise elucidation of the mechanisms of action that underlie the biocontrol effect as the present findings were based on few plant defense related genes but not the entire proteomic and transcriptomic data; (c) lack of post inoculation interval assessment for proper evaluation of the ePGPB drench windows to understand the effective timeline and durability of each strain; (d) quantification of the inoculated ePGPB strains that undergoes a biocontrol activity against both viruses and fungal pathogens respectively; (e) evaluation of the ePGPB strains interaction and their environmental impact on other non-target organisms; (f) some of the employed ePGPB strains demonstrated an opposite or fluctuating effect in comparison to the results obtained during the studies previously carried out by Passera (2017), for instance, the plants treated with the strain 255-7 gave higher concentration of CymRSV despite showing lower

symptoms severity. Whereas, the present study showed correlation in between them by lowering both the symptoms severity and concentration of CymRSV in host plant.

Despite these challenges, the results achieved during the doctoral study confirmed the previous conclusions and added valuable pieces of information in our understanding about the traits these ePGPB carried, particularly, in individuating different mechanism of action of the different strains in different host plants with or without the presence of pathogen. Providing growth promotion and protection against the pathogen on a broad range of host plants is one of the most essential components for the establishment of microbial based products in the market. Since the protocols for developing a plant protection product have different phases starting from laboratory to mass production, some of the factors are still not clear which must be investigated in the future studies, such as: (a) the timings of ePGPB treatments, pathogen inoculation and phenological stage of the host plant needs to be taken into consideration. For instance, while carrying out future trials against viruses, the duration of prior delivery of ePGPB treatments has to be determined in order to reach the maximum level of priming effect that would aid in limiting the replication of target virus within the host plant. Furthermore, it would be interesting to carry out bioassays using the same treatments on other plant species, of agricultural importance, which are susceptible to the viruses (natural host plants), to verify whether the effectiveness of the treatments is greater in species that are less sensitive to viruses than model host plant; (b) In order to launch the microbial product to the market, future trials should be done to determine their nature of action, such as, whether the use of ePGPB products can be preventive, curative, or both and which works best for selective pathogen and plant; (c) investigate *in planta* real-time imaging to visualize the movement and endophytic colonization pattern of investigated strains. Because of the strain's endophytic nature, the species to which they belong are easily found in the plant, thus not allowing the distinction between the ePGPB strains administered and the strains naturally present within the plant. For this reason, the investigated ePGPB strains should be labeled with fluorescent molecules to verify their adaptive capacity in host plant by confocal microscopy; (d) conducting the experiments that include host-pathogen-ePGPB interactions would be an asset and would provide a full picture of investigated strains; (e) carrying out the proteomics and/or transcriptomics analyses on plants that received both ePGPB strains and pathogen inoculum could better unveil the hallmarks of ISR facilitated by investigated ePGPBs; and most importantly, (f) carrying out the whole genome sequencing (WGS) to compare between control plants, infected plants, plants treated with ePGPB strains, and the plants inoculated with both ePGPB treatments and

pathogens would fill the gaps in defining the function of each of the bacterial members within the community weather they boost or alter some vital processes within the plant.

Regarding the second approach, several studies demonstrated the efficacy of using dsRNA as a key molecule in the induction of resistance in naturally-infected plants as well as in virus resistance via post-transcriptional gene silencing (Das and Sherif, 2020; Jadhav et al., 2019; Liu et al., 2008; Waterhouse et al., 1998). It's practical applicability have been shown positive outcomes in modern agriculture industry where dsRNA derived from viral genes have been utilized as nucleic acid pesticide protection in the field (Zotti et al., 2018). With this information as a starting point, in Chapter 2 of Aim 1, we attempted a preliminary study by using a non-transgenic approach to *in vitro* produce dsRNA derived from coat protein (CP) genes of *Tomato Aspermy Virus* (TAV) to induce resistance against TAV in *N. benthamiana* plants by exogenous application. Both inoculums (TAV and CP-derived dsRNA TAV) were made with two dilutions (1:5 and 1:10) using diluted solution buffer to better understand which dilution factor works better in terms of inducing systemic resistance. We examined the phenotypic traits looking for symptoms such as stunted growth and the presence of blisters, crinkling, leaf distortion, and systemic vein clearing at 6, 12 and 18 dpi. Our findings showed slight increment in plant heights treated with CP-derived dsRNA TAV at dilution 01:10 (6 and 12 dpi) but were not successful in reducing any of the above-mentioned symptoms.

Seeing how this setup gave some results, it might be possible that several factors were responsible for the incompetency to trigger post-transcriptional gene silencing derived from CP gene expression. One reason could be the concentrations used for dsRNA inoculum in the bioassay that might not be appropriate and were lower than required. Second reason that could explain the incompetency would be the use of the sequence which was not specific to the particular isolate of inoculated TAV, and the third reason could be the unstable nature of dsRNA as it might be degraded eventually at the time of inoculum preparation or in the delivery to the host plant that would cause this inefficacy. Such attributes have already been shown relevant limitations indicated in the past studies (Dalakouras et al., 2016; Dubrovina and Kiselev, 2019; Mitter et al., 2017a; Numata et al., 2014). Furthermore, the ecological competency of dsRNA, the conditions that can impede or enhance their performance in the field or greenhouse, and other barriers that can reduce their efficacy.

Despite such limitations, these preliminary results have opened new insights that will be useful enough to conduct future studies in an appropriate manner. Such as: (a) determination and application of the higher concentration of CP-derived dsRNA TAV inoculum would confer the RNAi efficacy against TAV infection in the plants; (b) utilization of more specific and suitable viral genes or optimization of *in vitro* dsRNA molecules production using higher size or more specific sequences would potentiate the future trials; (c) In addition, nanoparticles can be used as carriers of dsRNA for improving the stability and sustained release in the plants. The similar study was conducted by Mitter and colleagues (2017b), where the dsRNA derived from CP of CMV was produced using layered double hydroxide (LDH) nanosheets as carriers which forms Bioclay as these delay the degradation of dsRNA onto the tobacco leaf surface for up to 20 days in the presence of atmospheric CO^2 and moisture. Though the nanotechnology-based dsRNA delivery seemed quite expensive, but it showed durability and long-term protection; (d) Another promising bioassay that would be interesting to employ is the microbial-based dsRNA production using ePGPB strains (particularly, strain 260-02) as the combined form of protection might give extra-ordinary outcomes against the virus infection. By following the studies conducted by Niehl and colleagues (2018), where they used the components of bacteriophage phi6 to engineer stable and accurate in vivo dsRNA system in *P. syringae* bacteria against tobacco mosaic virus (TMV). Such production strategy allowed the production of high quality, long dsRNA molecules that enabled the broad application in an efficient, more flexible, non-transgenic and environment friendly way against viruses.

Since the general scope of the doctoral research activities was to implement new plant protection products (PPPs) to the market that are target specific and environment friendly. The two biocontrol-based approaches (dsRNA and ePGPBs) followed during the present doctoral studies are still under development but in a promising phase. The use of both previously mentioned approaches should go in parallel for future diseases management strategies to avoid the development of resistance. Eventually, the more arsenals we have to control plant diseases, the more successful will be the integrated management strategy.

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