The evolution of effector genes in oomycetes and biological control

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(Amirhossein Bahramisharif)

THE EVOLUTION OF EFFECTOR GENES IN OOMYCETES AND BIOLOGICAL CONTROL

SUMMARY

Phytophthora infestans, the Irish famine pathogen, is the most devastating pathogen of commercially important *Solanum* species, causing late blight. Several virulence factors are involved in pathogenesis. One of the important factors is the secretion of effector proteins by *P. infestans.* Some of the effectors contain the RXLR motif and are translocated into the host cell. The effector proteins regulate a pathogen's biochemical functions and therefore, cause infection. However, the host plant is able to detect pathogen's effector proteins known as avirulence/Avr by its resistance proteins (R protein). The RXLR genes have highly diverse amino acid sequences within/between species that may have been caused by gene duplication, gene loss or diversifying selection. The interaction between plant and pathogen is, therefore, complex and requires knowledge of the RXLR genes involved and their evolution.

This study investigated the molecular evolution of several Avr genes, core effector genes and RXLR genes that are predicted to have a nuclear localization signal (NLS) motif in *Phytophthora* clade 1C species, including *P. infestans, P. ipomoeae* and *P. mirabilis*. Interestingly, the number of RXLR genes detected in *P. infestans* was significantly larger than in *P. ipomoeae* and *P. mirabilis*. Protein structural changes, caused by a single nucleotide polymorphism, were also observed in the RXLR genes. This is important because changes in a protein's structure will most likely lead to changes in function. Different patterns of expression were found in RXLR genes evaluated during infection of *Solanum* species. Furthermore, the current study detected 11 RXLR genes, including Avr and NLS genes that evolved under diversifying selection. This approach provides knowledge towards breeding for late blight resistance and long-term management.

Late blight management is very challenging. The current study investigated the influence of non-chemical products such as a compost and biological agents on tomato resistance to late blight, because the continuous application of synthetic fungicides may pose a huge risk to human and the environment. Evaluation of tomato growth and

resistant to late blight showed that plants treated with *Bacillus subtilis* subsp. *subtilis* and *Trichoderma harzianum* were significantly bigger and more resistant to *P. infestans*. However, the commercial products including FZB24 and FZB42 were less effective at tomato growth and protection. The combination of oak–bark compost and bio-agents was also investigated and showed that only oak–bark compost combined with *B. subtilis* subsp. *subtilis* treatment was consistent and effective in late blight management. In contrast, the combined compost and *T. harzianum* treatment caused a significant increase in late blight severity.

This study has improved our knowledge on the biology and the evolution of *Phytophthora* species, and has identified biological control strategies for use to enhance tomato resistance and suppress late blight. Oomycetes are immensely important pathogens and can cause significant crop losses. This study provided insights into the evolutionary mechanisms by which *Phytophthora* gained the ability to infect tomato. Furthermore, difficulties in disease management were investigated and showed the need for integrated management strategies. The knowledge gained in this study can be used to enhance soil nutrient status, increase yield in organic agriculture and develop disease-resistant crops.

DIE EVOLUTION VON EFFEKTOR-GENEN BEI OOMYZETEN UND METHODEN FÜR DEN BIOLOGISCHEN PFLANZENSCHUTZ

ZUSAMMENFASSUNG

Phytophthora infestans, der Verursacher der irischen Hungersnot zwischen 1845 und 1849, ist das bedeutendste Pathogen kommerziell wichtiger Arten der *Solanaceae* und der Erreger der Krautfäule. An der Pathogenese sind mehrere Virulenzfaktoren beteiligt. Einer davon ist die Ausscheidung von Effektorproteinen durch *P. infestans*. Effektorproteine die über ein RXLR-Motiv verfügen, werden in die Zellen des Wirts verlagert. Effektorproteine regulieren die biochemischen Funktionen des Pathogens und verursachen damit die Infektion. Die Wirtspflanze ist in der Lage einige Effektorproteine über spezifische Resistenzproteine (R-Protein) zu erkennen. Diese Effektoren werden als Avirulenzproteine (AVR-Proteine) bezeichnet. Die Aminosäuresequenzen der RXLR Gene sind sowohl innerartlich als auch zwischen Arten äußerst divers, was durch Genduplikation, Genverlust oder diversifizierende Selektion verursacht worden sein könnte. Die Interaktion zwischen Pflanze und Pathogen ist daher komplex. Tiefergehendes Verständnis dieses Prozesses erfordert somit Wissen über die beteiligten RXLR-Gene sowie deren Evolution.

In dieser Arbeit wurde die molekulare Evolution mehrerer Avr-, RXLR- und Core-Effektorene, von denen angenommen wird, dass sie ein Kernlokalisierungssignal (nuclear localization signal, NLS) aufweisen, analysiert. Hierzu wurden Arten der *Phytophthora* Klade 1C, welche *P. infestans*, *P. ipomoeae* und *P. mirabilis* umfasst, untersucht. Interessanterweise war die Anzahl der RXLR-Gene, die bei *P. infestans* gefunden wurden, signifikant höher als bei *P. ipomoeae* und *P. mirabilis*. Außerdem wurden bei den RXLR-Genen Veränderungen in der Proteinstruktur beobachtet, welche durch einen einzelnen Nukleotid-Polymorphismus verursacht wurden. Dies ist bedeutsam, da Veränderungen in der Proteinstruktur in der Regel Veränderungen in der Funktion nach sich ziehen. Bei der Analyse der Expression von RXLR-Genen während der Infektion von Arten der Gattung Solanum, wurden unterschiedliche Expressionsmuster gefunden. Weiterhin wurden im Rahmen dieser Arbeit 11 RXLR-Gene entdeckt, darunter Avr- und NLS-Gene, welche unter diversifizierender Selektion entstanden sind. Die gesammelten Erkenntnisse können bei der Züchtung gegen

Krautfäule resistenter Sorten und bei der langfristigen Bekämpfung von Krautfäule Anwendung finden.

Die Bekämpfung von Krautfäule ist sehr herausfordernd. In dieser Arbeit wurde der Einfluss nicht-chemischer Produkte, wie z. B. Kompost und biologischer Wirkstoffe, auf die Resistenz von Tomaten gegen Krautfäule untersucht, da der stetige Einsatz synthetischer Fungizide ein großes Risiko für Menschen und Umwelt darstellen könnte. Bei der Analyse des Wachstums von Tomaten und deren Resistenz gegenüber Krautfäule zeigte sich, dass Pflanzen, die mit *Bacillus subtilis* subsp. *subtilis* und *Trichoderma harzianum* behandelt wurden, signifikant größer wurden und verstärkte Resistenz gegenüber *P. infestans* aufwiesen. Bei Anwendung von kommerziell erhältlichen Produkten, darunter FZB24 und FZB42, war der positive Effekt auf das Wachstum der Tomaten sowie die Wirksamkeit bei deren Schutz weniger stark ausgeprägt. Untersucht wurde auch die kombinierte Anwendung von Eichenrinden-Kompost und Mikroorganismen für den biologischen Pflanzenschutz. Dabei zeigte sich, dass ausschließlich die kombinierte Anwendung von Eichenrinden-Kompost und *B. subtilis* subsp. s*ubtilis* langfristig und effektiv Krautfäule unterdrückt. Bei Anwendung von Kompost und *T. harzianum* kam es hingegen zu einer signifikanten Zunahme in der Aufprägung von Krautfäule.

Diese Arbeit hat unser Wissen über die Biologie und Evolution von Arten der Gattung *Phytophthora* verbessert und Strategien zur biologischen Schädlingsbekämpfung, zur Verbesserung der Resistenz von Tomaten sowie zur Unterdrückung von Krautfäule identifiziert. Oomyzeten sind überaus bedeutende Pathogene und können erhebliche Ernteverluste verursachen. Diese Arbeit bietet Einblicke in die evolutionären Mechanismen, über die *Phytophthora* die Fähigkeit erlangte Tomaten zu infizieren. Schwierigkeiten bei der Schädlingsbekämpfung wurden aufgezeigt, was den Bedarf an ganzheitlichen Bekämpfungsstrategien verdeutlichte. Die Erkenntnisse, die im Rahmen dieser Arbeit erlangt wurden, können dazu angewendet werden, den Nährstoffgehalt von Böden zu verbessern, den Ertrag in der biologischen Landwirtschaft zu steigern und krankheitsresistente Nutzpflanzen zu entwickeln.

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“This dissertation is dedicated to the memory of my brother, Masoud, who always believed in my success”

CONTENTS

CHAPTER 1

CHARACTERIZATION AND MANAGEMENT OF *PHYTOPHTHORA INFESTANS* IN TOMATOES

1.1 Introduction

Tomato (*Solanum lycopersicum* L.) is a commercially important crop that is grown across the world (Fao.org). Tomato has several benefits and it is loaded with antioxidants (Abushita et al. 1997; Frusciante et al. 2007). Production of tomato cultivars is, however, limited by several biotic and abiotic factors. Late blight disease is one of the important biotic factors that is caused by the infamous potato famine pathogen, *Phytophthora infestans* (Mont.) de Bary (Agrios 2005; Fry et al. 2015).

Phytophthora infestans is an oomycete pathogen that commonly infects potatoes and tomatoes in cold and wet conditions (Harrison 1992). The lesions occur on aboveground tissues of tomatoes (Fry et al. 2015). This oomycete is able to initiate infection by secretion of numerous effector proteins (Kamoun 2006; Schornack et al. 2009). Some of these effector proteins are translocated into the host, using the RXLR motif (Kamoun 2006; Schornack et al. 2009). On the other side, a host immune response to the pathogen can be activated and this often leads to a hypersensitive response and the induction of cell death (Kamoun 2006).

Late blight management in tomato requires integrated management. This can include the use of chemical fungicides, cultural practices and biological control. Synthetic fungicides has been shown good results against oomycete pathogens, however these chemicals cannot always be applied due to the development of fungicide resistance and acute toxicity or by organic farmers (Taylor et al. 2002; Schummer et al. 2012a; Schummer et al. 2012b; Matson et al. 2015). Therefore, alternatives to fungicides such as the use of organic amendments and biological agents or the combination of these treatments have become very popular (Hoitink et al. 1997; Termorshuizen et al. 2006; Kim et al. 2007; Bahramisharif et al. 2013a; Tewoldemedhin et al. 2015; Yao et al. 2016; O'Brien 2017; Bahramisharif and Rose 2019).

The aim of this review is to provide an overview of tomato production, the importance of *P. infestans*, late blight disease and virulence factors, and management strategies for sustainable agriculture.

1.2 Tomato

Tomato is one of the major agricultural crops that is native to South America. Tomato is grown worldwide with the global fruit production of 100 million tons (Fao.org). Although tomato can grow in different climates and conditions, the ideal temperature for growth is between 18 to 25 °C. China is the world's leading producer of tomato, followed by India and the USA (Fao.org).

Tomato is the main dietary source of nutrients and has potent antioxidants properties such as carotenoids (beta-carotene and lycopene), vitamin C, vitamin E and phenol compounds (Abushita et al. 1997; Frusciante et al. 2007). Lycopene is a red carotene that has been reported to have inhibitory effects on cancer and heart disease (van Breemen and Pajkovic 2008; Böhm et al. 2012).

Several varieties of tomato are cultivated worldwide, including beefsteak tomato, Campari tomato, cherry tomato, grape tomato, plum tomato and Tomberry tomato. The production of tomatoes is however difficult, because the plants are very susceptible to several plant pathogens. The main pathogens include *Alternaria solani* Sorauer, *Fusarium oxysporum* Schlecht. emend. Snyder and Hansen, *Phytophthora infestans,*

Septoria lycopersici Speg., *Ralstonia solanacearum* Smith and *Verticillium dahliae* Kleb. (Mills et al. 2002; Fry et al. 2015; Adhikari et al. 2017; Akaeze et al. 2017; Papadaki et al. 2017; Shutt et al. 2018). Of these, *P. infestans* is the most devastating pathogen that can severely damage the leaves, stems and fruits of tomato.

1.3 *Phytophthora infestans*

1.3.1 Biology

The genus *Phytophthora* contains hundreds of known and unknown species. One important species is *P. infestans* that is known as one of the most aggressive and destructive plant pathogens, causing potato and tomato late blight in the last hundred years (Agrios 2005; Fry et al. 2015). This pathogen belongs to the phylum Oomycota, class oomycetes, order Peronosporales and family Peronosporaceae. *Phytophthora infestans* is identified as a fungus-like organism, because its cell wall structural component is different compared to a true fungus. The primary components in *Phytophthora* cell wall structure consist of cellulose and glucans, whereas a cell wall in fungi contains a high proportion of chitin (Aronson et al. 1967; Bartnicki-García 1968; Erwin and Ribeiro 1996). Furthermore, unlike fungi that have haploid thallus in most phases of their life cycle, the vegetative nuclei in *Phytophthora* species is diploid (Brasier and Sansome 1975; Kamoun 2003; Govers and Gijzen 2006).

Phytophthora infestans include sexual and asexual stages. During sexual reproduction, the fusion of sexual structures (antheridia and oogonia) can develop a thick-walled structure called an oospore (Schumann and D'Arcy 2000). Oospores are important for their role in overwintering and survival. However, sexual reproduction may only occur when the two mating types (A1 and A2) coexist, because *P. infestans* is heterothallic (Fry et al. 2015). Asexual reproduction occurs through the formation of sporangia and/or zoospores, depending on the weather conditions (Schumann and D'Arcy 2000). In warmer conditions, sporangia are produced by sporangiophores which can be dispersed by air or physical contacts. However, in cool and wet conditions, zoospores are released by sporangia and they are able to swim towards the host plant (Fry et al. 2013). Zoospores can germinate via a germ tube and penetrate the host

epidermis. In general, asexual reproduction leads to a rapid proliferation of the pathogen (Fry et al. 2015).

1.3.2 Epidemiology

Late blight can be affected by several environmental factors. One important factor is moisture. At high consistent humidity, sporangia are formed on the lower foliage and stems (Drenth et al. 1995; Cohen et al. 1997). Another important factor in the epidemiology of disease is temperature. The optimal temperature for growth and germination of sporangia is 18 to 21 °C (Becktell et al. 2005). Although *P. infestans* is able to spread in different conditions, it is known to be more devastating under cooler and wet conditions. In a preferable condition, *P. infestans* only requires a few days to demolish the entire field of potato (Fry 2008).

1.3.3 Origin of *Phytophthora infestans*

The population dynamics and population shifts especially in new virulent genotypes are very important. Although several studies have shown that most losses in the 1990s were caused by a new genotype of *P. infestans* (Fry and Goodwin 1997; Goodwin 1997), the origin of the Irish famine strain appears to be complex. In 1950s, *P. infestans* was thought to have originated from central Mexico, because the sexual form was discovered in Mexican populations of *P. infestans* (Niederhauser 1956; Galindo and Gallegly 1960). Prior to that time, *P. infestans* was believed to have only asexual reproduction (Reddick 1939). Although a few studies on the evolutionary history of *P. infestans* and, a hybrid species, *Phytophthora andina* Adler and Flier, sp. nov. have questioned this assumption (Martin et al. 2015), an extensive sequence analysis of *P. infestans* and all the closest relatives provided evidence that *P. infestans* might have originated from central Mexico (Goss et al. 2014).

1.3.4 Pathogenicity and virulence

Many plant pathogens including *Phytophthora* species secrete effector proteins that modulate host responses and have been implicated in pathogenesis (Tyler et al. 2006). Some of these effectors, called avirulence proteins (Avr), are recognized by a

corresponding plant resistance gene and therefore activate hypersensitive response (HR) in host cells which leads to disease inhibition. The *Phytophthora* genomes contain hundreds of effectors (Tyler et al. 2006; Haas et al. 2009). These effectors undergo extensive gene amplification and loss in genomes during evolution that contribute to virulence (Haas et al. 2009; Qutob et al. 2009). Furthermore, these effectors show high rates of sequence divergence, providing strong evidence for diversifying selection (Allen et al. 2004; Liu et al. 2004; Win et al. 2007).

Oomycete effectors are grouped into two main categories including apoplastic and cytoplasmic effectors, according to their sites of action in plant cell (Kamoun 2006; Whisson et al. 2007; Schornack et al. 2009). Several oomycete effectors include an important class of cytoplasmic effectors, contained a RXLR motif in the N-terminal region, followed by the C-terminal domain (Haas et al. 2009). The N-terminal region delivers effector proteins to plant cell. The C-terminal domain plays an important role in regulating protein function and determines the biological activities (Kamoun 2006; Schornack et al. 2009).

1.3.5 Structure of RXLR effector proteins

The structure of a protein defines key mechanisms involved in molecular interactions and determines the biochemical functions (Alberts et al. 2002; Ye et al. 2015). However, changes in the amino acid sequence of a protein caused by random mutations may result in different biological activities in a cell (Alberts et al. 2002; Bhattacharjee and Biswas 2013). Therefore, the analysis of protein secondary structure can provide insights into the protein function (Pollastri et al. 2002; Karypis 2006). In recent years, several methods have been developed for the prediction of protein secondary structure (Feng et al. 2014; Ye et al. 2015; Kieslich et al. 2016; Li et al. 2017; Yavuz et al. 2018; Panja et al. 2019).

The secondary structure of an effector protein refers to the three-dimensional shape that is formed through Coulomb forces by hydrogen bonds linking amide hydrogens and carbonyl oxygens groups in the backbone chain (Gromiha 2010). The secondary structure consists of mainly α-helices and β-structures, but other elements such as a turn and a random coil can also be found (Berg 2002; Gromiha 2010; Feng et

al. 2014). Interestingly, a random-coil conformation can occur when a protein lacks the regular secondary structure (Feng et al. 2014).

1.3.6 Identification and detection

The identification of *P. infestans* can be performed by morphological characteristics and molecular techniques. Several morphological characters such as mycelial growth, hyphal swellings, the presence of sporangia and oogonia, length and breadth of sporangia, shape of antheridia and oogonia, growth rates and optimal temperatures have been used to identify oomycete species (Erwin and Ribeiro 1996). However, morphological identification of *Phytophthora* species can be time consuming and difficult, due to intraspecific variability and overlapping in morphological characters, and problems with sporulation (Ristaino 1990; Brasier 1991; Erwin and Ribeiro 1996; Hantula et al. 2000). Therefore, the use of molecular techniques is required for an accurate identification and detection of oomycetes.

Molecular biology tools such as enzyme-linked immunosorbent assay (ELISA), amplification of DNA using the polymerase chain reaction (PCR) and alloenzyme markers have been developed for the detection and identification of *P. infestans* (Goodwin et al. 1995; Trout et al. 1997). Most recently, Blair et al. (2008) identified multi-locus genetic markers to construct a phylogeny of 82 *Phytophthora* species for species identification. For the amplification of DNA sequence, the internal transcribed spacer (ITS) region has proven effective for screening fungal and oomycete species (Willits and Sherwood 1999; Cooke et al. 2000; Silvar et al. 2005). In addition to the ITS region, ß-tubulin and the mitochondrially encoded regions such as cytochrome *c* oxidase subunit I and II (*COX1* and *COX2*) have been reported to be valuable for the detection and taxonomy of oomycete species, including *P. infestans* (Bala et al. 2010; Bahramisharif et al. 2013b; Bahramisharif et al. 2014; de Vries et al. 2017; Bahramisharif and Rose 2019).

1.4 Management strategies

The management of late blight is complex, due to a rapid reproduction cycle and presence of new genotypes. In the last few decades, several control techniques have

been evaluated for the disease reduction, including chemical methods, cultural practices and biological control.

1.4.1 Chemical fungicides

The application of fungicides is very common worldwide, especially in developing countries. Chemical fungicides are considered one of the most effective disease management methods. The commercial fungicides such as mancozeb + metalaxyl and propamocarb + fluopicolide have been reported to be effective in reducing mycelial growth of *Phytophthora* species (Mendonca et al. 2015). Furthermore, a study by Pirondi et al. (2017) evaluated several fungicide classes and showed that cyamoxanil (cyanoacetamide oxime), dimethomorph (carboxylic acid amides) and metalaxyl-M (phenyilamide) were able to suppress late blight of tomato. Although phenylamide fungicides are in commercial use and have been shown effective at suppressing *P. infestans* (Saville et al. 2015), metalaxyl resistance in the pathogen population has been reported in different regions (Diriwächter et al. 1987; Matson et al. 2015; Chen et al. 2018).

1.4.2 Cultural practices

Cultural practice is a traditional method in farming and is important for improving soil fertility, moisture conservation, crop yield and disease suppression. Water plays an important role in agriculture, but several oomycetes such as *P. infestans* prefer wet conditions. Considering the pathogen life cycle and the release of zoospores, water management is critical (Erwin and Ribeiro 1996).

Soil nutritional status can also affect disease severity and incidence. Calcium is an essential nutrient that is required by plants. The use of calcium has been reported to improve plant growth and reduce oomycete disease severity, including late blight of potato (Maloney et al. 2005; Benson et al. 2009; Seifu 2017). Similarly, the application of phosphite compounds such as calcium phosphite and potassium phosphite have proven effective in protecting potato tubers from *Fusarium solani* (Mart.) Sacc., *P. infestans* and *Rhizoctonia solani* J.G Kühn, due to plant cell wall reinforcement and

regulation of defense response (Lobato et al. 2008; Machinandiarena et al. 2012; Olivieri et al. 2012; Lobato et al. 2018).

In organic farming, composts have gained popularity for use as a soil conditioner or a fertilizer. Composts are made of various organic materials that contain a broad range of beneficial microorganisms including actinomycetes, bacteria, endophytic fungi and protozoa (Keeling et al. 1995; Craft and Nelson 1996; Bahramisharif and Rose 2019). These microbes are required for the composting process that is typically occurred in three stages: the mesophilic, the thermophilic and the maturation (Partanen et al. 2010).

The microbial community in a compost also play an important role in plant growth promotion and disease suppression (Diab et al. 2003; Noble and Coventry 2005; Gutierrez-Miceli et al. 2007; Kim et al. 2007; Miles et al. 2012). Intriguingly, Craft and Nelson (1996) found a reduction in oomycete disease severity in different batches of compost with higher microbial biomass and activity. However, the effect of compost may vary widely according to the physical, chemical and microbial properties (Termorshuizen et al. 2006).

Another important cultural practice is to grow dissimilar crops in rotation which is also required in organic farming. Crop rotation may prevent or reduce a population of pests, pathogens and weeds (Bahramisharif et al. 2013a; Leoni et al. 2013; Finckh et al. 2015). A study by Aav et al. (2015) found that crop rotation is important for destroying oospores of *P. infestans*. The use of different crops in sequence can also help replace essential nutrients and prevent soil erosion. For example, nitrogen-fixing crops such as legumes can help maintain nutrient balances in the soil and increase sustainability (Havlin et al. 1990; Fry 2012).

1.4.3 Biological control

Biological control is considered one of the main strategies for improving sustainable crop production and protection (Yan et al. 2002; Kim et al. 2007; An et al. 2010; Horner et al. 2012; Chowdappa et al. 2013; Kabir et al. 2013; Yao et al. 2016; O'Brien 2017; Bahramisharif and Rose 2019). Four forms of biological control are

recognized. These include classical, inoculation, inundation and conservation (Eilenberg et al. 2001). Inoculation and inundation biological control have been developed for integrated pest management (IPM).

Biological control agents (bio-agents) can be found in many places on Earth, but the majority of them live in the soil. Several bio-agents have been evaluated for their ability to enhance plant growth and suppress pathogens, including tomato growth and late blight suppression (Kabir et al. 2013; Bahramisharif and Rose 2019). Some of the most common bio-agents belong to the genus *Bacillus* and *Trichoderma*; both of these are defined as plant growth promoting agents. The main modes of action for growth promotion and disease suppression include regulation of phytohormones and expression of induced systemic resistance (ISR) (Howell 2003; Beneduzi et al. 2012; Pieterse et al. 2014). The efficacy of bio-agents is, however, not always consistent due to the structure and complexity of the soil.

1.5 Conclusion

Cultivated tomato is a major agricultural crop and is highly susceptible to late blight caused by *P. infestans. Phytophthora infestans* secretes an array of RXLR genes to facilitate infection. These effectors may be recognized by the host resistance proteins that trigger defense response. However, through the action of natural selection, some pathogens have overcome host resistance. It is therefore, important to understand the function and evolutionary history of pathogen effectors in this devastating pathogen.

The management of late blight is very complex and difficult, especially in organic farming. Although the use of biocidal chemical compounds has been shown effective in the reduction of the disease, fungicides cannot be used in organic production and may lead to major environmental problems. Therefore, alternative management strategies to fungicide treatments such as the use of organic amendments and biocontrol agents could provide promising practices in late blight management.

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CHAPTER 2

MOLECULAR EVOLUTION OF RXLR GENES IN THE IRISH FAMINE PATHOGEN *PHYTOPHTHORA INFESTANS* AND CLADE 1C RELATIVES

Status	In preparation
Citation	Bahramisharif A, Shakya SK, Knaus BJ, de Vries S, Judelson HS, Grünwald NJ, Rose LE (2019) Molecular evolution of RXLR genes in the Irish famine pathogen *Phytophthora infestans* and clade 1C relatives
Own contribution	70% Designed and conceived research Conducted the experiments Analyzed the data Interpreted the data Wrote the manuscript

2.1 Abstract

The Irish potato famine *Phytophthora infestans* is considered to be one of the most devastating plant pathogens. This economically destructive pathogen can rapidly evolve new virulent genotypes because of its large effective population size and world-wide distribution. The genome of *P. infestans* harbors hundreds of effectors that contribute to virulence and accelerate fatal infection of the host plant. One of the main families of effector proteins is characterized by the presence of an RXLR domain, which is required for translocation inside the host cell. However, little is known about the evolution of these effectors. Therefore, to gain greater insight into the mechanisms involved in plant-pathogen interactions, the molecular evolution of avirulence (Avr) genes, core effector genes and RXLR genes with a nuclear localization signal (NLS) motifs from three *Phytophthora* clade 1C species was evaluated. Our analysis of the presence and absence in RXLR genes across this group shows that *Phytophthora ipomoeae* and *Phytophthora mirabilis* have fewer RXLR genes compared to *P. infestans.* Furthermore, all three clade 1C species have only two Avr genes (*Avrvnt1* and *AvrSmira1)* in common. In general, a higher nucleotide diversity was observed in the RXLR genes compared to core orthologous genes. In addition, some of the substitutions are predicted to alter the secondary structure of the protein which may affect protein function. The RXLR effectors also showed different expression patterns during infection of *Solanum* species. Finally, based upon population genetic analyses, 11 genes showed evidence of recent diversifying selection. A deeper understanding of these effectors could provide efficient and novel strategies for long-term disease management and developing pathogen-resistant crops.

2.2 Introduction

The Irish famine pathogen, *Phytophthora infestans* (Mont.) de Bary causes serious yield losses on potato and tomato (Agrios 2005; Fry et al. 2015), making this pathogen a formidable threat to two important crops. This pathogen belongs to the phylum Oomycota and is known to cause late blight. *Phytophthora infestans* interacts with plants in two sequential phases: first as a biotroph and then as a necrotroph (Whisson et al. 2007). In suitable conditions, *P. infestans* is capable of destroying entire

fields of crops in a short period of time, due to a rapid asexual life cycle (Fry 2008; Fry et al. 2015).

Phytophthora infestans is closely related to *Phytophthora ipomoeae* Flier and Grünwald and *Phytophthora mirabilis* Galindo and H. R. Hohl and all three species are clustered within clade 1C of the genus *Phytophthora*. These species are known to have coexisted in central Mexico, their center of origin (Flier et al. 2002; Grünwald and Flier 2005; Goss et al. 2014). Unlike *P. infestans, P. ipomoeae* and *P. mirabilis* have not been reported to cause blight symptoms on *Solanum* species. *Phytophthora ipomoeae* has been reported to cause leaf blight on *Ipomoea longipedunculata* L. (the morning glory), while *P. mirabilis* has been reported to damage foliage of *Mirabilis jalapa* L. (four o'clock flower) (Goodwin and Fry 1994; Flier et al. 2002).

Plants and oomycete pathogens are involved in a chain of events, during their interactions. Most plants constitutively express innate immune receptors, also called plant pathogen recognition receptors or PRRs (Jones and Dangl 2006; Katagiri and Tsuda 2010; Cooke et al. 2012). At the time of attack, these receptors are able to recognize pathogen/microbe associated molecular patterns or PAMPs/MAMPs that trigger immune response called PAMP-triggered immunity (PTI) (Jones and Dangl 2006; Katagiri and Tsuda 2010; Cooke et al. 2012). In turn, many plant pathogens including oomycetes have evolved to modulate PTI by secreting effector proteins that are required for pathogenesis (Tyler et al. 2006). However, effector molecules can be recognized by specific plant resistance (R) proteins. The interaction between effector proteins and their cognate R proteins may lead to an immune response called "Effector Triggered Immunity" (ETI) which may result in a hypersensitive response (HR) at the site of infection. Effectors that lead to ETI in the host are referred to as avirulence (Avr) proteins. Over time, evolutionary changes in Avr genes allow pathogens to avoid recognition by their hosts R-proteins. These changes can include the loss of an Avr gene, the acquisition of a premature stop codon leading to a truncated protein or amino acid changes resulting in the loss of recognition by the cognate R gene and/or loss of expression of the Avr gene (Armstrong et al. 2005; van Poppel et al. 2008; Gilroy et al. 2011).

Oomycete effectors can be divided into two main categories: apoplastic and cytoplasmic effectors. Apoplastic effectors act outside the plant cells (intercellular space) and cytoplasmic effectors function inside plant cells following translocation through the haustorium (Kamoun 2006; Whisson et al. 2007; Schornack et al. 2009). One prominent class of cytoplasmic effector proteins is characterized by a conserved RXLR motif in the N-terminal region which can be found in many oomycete effectors (Haas et al. 2009). Although, over 500 RXLR genes have been reported from the genome of *P. infestans* strain T30-4 (Haas et al. 2009), only a few genes have been shown to have Avr activity (van Poppel et al. 2008; Champouret et al. 2009; Oh et al. 2009; Bos et al. 2010; Gilroy et al. 2011; Rietman et al. 2012; Du et al. 2018). The Avr genes have been a primary focus in the development of late blight resistant potatoes (Jo et al. 2014). Due to their important role in pathogenicity and virulence, RXLR genes have attracted the attention of many plant pathologists. While it is known the RXLR motif is necessary for translocation inside the host cell (Kamoun 2006; Schornack et al. 2009), the evolutionary history and diversification of this class of proteins has not been elucidated. Furthermore, there is no information on the presence or diversity of RXLR genes in *P. ipomoeae* and *P. mirabilis.* Therefore, the overall goal of this study was to determine the patterns of molecular evolution of RXLR genes in clade 1C of *Phytophthora* to gain a better insight into the mechanisms involved in plant-pathogen interactions. The aims of this study were to (1) identify a core set of effector genes conserved within *Phytophthora* species in clade 1C, (2) identify RXLR genes with a nuclear localization signal motif, (3) determine the putative impact of genetic variation on protein function in terms of secondary structure, (4) investigate genes with signatures of diversifying selection and (5) evaluate patterns of gene expression of *P. infestans* in cultivated and wild *Solanum* species during infection.

2.3 Materials and Methods

2.3.1 Data collection and sequencing

Raw genome sequence data of 12 strains of *P. infestans* and one strain of *P. mirabilis* were downloaded from GenBank (https://www.ncbi.nlm.nih.gov; Table 1). In addition, 10 isolates of *P. infestans,* four isolates *P. ipomoeae* from Mexico and three

isolates of *P. mirabilis* from Mexico were sequenced using the Illumina Hiseq3000 technology (Illumina, San Diego, CA, USA).

2.3.2 Gene mapping

Read libraries were mapped onto the *P. infestans* reference genome (T30-4) using Burrows-Wheeler Aligner (BWA) v0.7.13 with default settings (Li and Durbin 2009). Next-generation sequencing (NGS) coverage was evaluated by displaying the number of reference bases that are covered by mapped sequencing reads at various depths. To determine the rate of evolution, NGS data of 30 isolates were used for single-nucleotide polymorphism (SNP) discovery and genotype calling using GATK HaplotypeCaller v. 4.0.11.0 (DePristo et al. 2011). For analysis of phased haplotypes, genotype calling and haplotype estimation were performed using BEAGLE v4.1 under default settings (Browning and Browning 2007; Browning and Browning 2013). The gene sequences were reconstructed using the function "vcfR2DNAbin" implemented in the "vcfR v.1.6.0" package (Knaus and Grünwald 2017).

2.3.3 Breadth of Coverage and presence/absence polymorphism

Breadth of Coverage (BOC) was used as a metric for gene presence/absence polymorphisms (Cooke et al. 2012; Yoshida et al. 2013). BOC was defined as the proportion of positions sequenced in an isolate, at least once, compared to the reference genome. First, the coverage for each gene was calculated and divided by the length of gene sequence to get a BOC value. A BOC cutoff value of 0.90 was used to call RXLR gene presence/absence polymorphisms, because at this threshold the numbers of RXLR genes in *P. ipomoeae and P. mirabilis* were comparable to other *Phytophthora* species (Haas et al. 2009).

2.3.4 Haplotype diversity

To calculate the number of nucleotide haplotypes and diversity, a subset of validated Avr gene sequences were used. The number of observed haplotypes were calculated for each Avr gene by species using the R package "pegas v.0.10" (Paradis

2010). Then, the haplotype count was used to calculate haplotype diversity for each Avr gene, if present (Nei 1978).

2.3.5 Nucleotide diversity

Nucleotide diversity was calculated for 218 RXLR genes that were present in all three clade 1C *Phytophthora* species at a BOC cutoff value of 0.90. This value was further compared to the nucleotide diversity value of 218 randomly selected core orthologous genes. Nucleotide diversity was determined using the R package "pegas v.0.10" (Paradis 2010). To determine if the distribution of nucleotide diversity differed between RXLR and core orthologous genes, a non-parametric Kruskal-Wallis test was conducted in R (R Core Team 2014). The value of nucleotide diversity was also evaluated for the 343 RXLR genes that showed presence/absence polymorphism and for 220 conserved RXLR genes. Nucleotide diversity was also calculated for synonymous sites and nonsynonymous sites separately using DnaSP v6 (Rozas et al. 2017).

2.3.6 Nuclear localization signal prediction

All the RXLR genes were selected from a study by Haas et al. (2009). To identify a nuclear localization signal (NLS) motif in the genes, NLStradamus was used (Ba et al. 2009). NLStradamus uses a simple hidden Markov model to predict novel NLSs in proteins.

2.3.7 Protein secondary structure

To determine the putative impact of genetic variation on protein function, comparative methods were used. Predicted protein sequence alignments of Avr genes and genes with NLS motifs were inferred using the Geneious plugin ClustalW 2.1. Secondary protein structures were predicted using EMBOSS 6.5.7 tool by implementing Garnier Osguthorpe Robson algorithm (Garnier et al. 1978; Rice et al. 2000). The differences in predicted secondary structures were compared and the underlying causative nucleotide substitutions were identified. Mann-Whitney U test

was used to calculate significant differences between classes of structural elements (Mann and Whitney, 1947).

2.3.8 Gene expression

To investigate regulation of gene expression of five RXLR-NLS genes during infection with *P. infestans* D12-2, gene-specific primers were designed using the Geneious plugin Primer 3 2.3.7 (Rozen and Skaletsky 1999) (Table 2). Using reverse transcription polymerase chain reaction (RT-PCR), the transcript abundance of a subset of RXLR genes, as well as the cytochrome *c* oxidase subunit II (*COX2*) was determined for *Solanum lycopersicum* and *S. pimpinellifolium* at six time points (0 hpi, 6 hpi, 24 hpi, 48 hpi, 72 hpi, 96 hpi) following inoculation with *P. infestans* strain D12-2. The RT-PCR reactions were carried out in a total volume of 20 µl consisting of Green GoTaq® Flexi Buffer, 2U GoTaq® Flexi DNA Polymerase (Promega, Fitchburg, WI, USA), 1.25mM $MgCl_2$, 0.1 mM dNTPs and 0.2 mM of each primer. The T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) was used for amplification of *P. infestans* with the following PCR conditions: initial denaturation at 95°C for 3 min, 34 cycles of 95°C for 30 s, annealing for 30 s at 60°C, extension at 72°C for 1.30 min, and final extension at 72°C for 5 min. PCR products were run on a 1% agarose gel and DNA was stained using Midori Green Advance (Nippon Genetics Europe, Dueren, Germany).

2.4 Results

2.4.1 Sequencing and mapping

The majority of short sequencing reads from *P. infestans*, *P. ipomoeae* and *P. mirabilis* could be aligned to the *P. infestans* T30-4 reference genome (Supplemental fig. S1). Under a BOC cutoff value of 90%, 220 RXLR genes were detected in samples from all three species of *Phytophthora*. As expected, higher BOC cutoff values resulted in a lower number of genes retained (Supplemental fig. S2). For example, at a BOC cutoff of 0.95, only 177 core effector genes were detected in all three species of clade 1C *Phytophthora*. The number of core RXLR effectors detected at a BOC cutoff value of 0.90 in each genome were: 333 for *P. ipomoeae,* 353 for *P. mirabilis and* 397 for *P. infestans*.

2.4.2 RXLR gene coverage in *Phytophthora* species

The coverage of RXLR genes within each genome relative to the *P. infestans* reference genome (T30-4) was evaluated. The greatest coverage was found in the US-23 lineage, followed by the Mexican isolate, P10650. The genomes of individuals of *P. infestans* from the Mexican sample showed lower gene coverage compared to the genomes from other individuals of *P. infestans* (Supplemental fig. S3). The coverage of RXLR genes was the lowest for *P. mirabilis* and *P. ipomoeae* (Fig 1).

2.4.3 Presence/absence variation

The genes displaying presence/absence variation (343 genes) were distributed across 106 RXLR gene families out of 252 RXLR families described in Haas et al. (2009). The largest number of genes with presence/absence variation belonged to the RXLR family 1 (42/85), followed by RXLR family 5 (21/22), RXLR family 9 (16/17) and RXLR family 7 (15/18) (Supplemental table S1). RXLR family 1 does not contain any known Avr genes, however the RXLR families 5 and 7 contain *Avr-blb2* and *Avr2* genes and their paralogs.

The pattern of presence/absence variation was also investigated for well characterized Avr genes in *P. infestans* (Fig. 2). In total, two Avr genes, including *AvrSmira1* (*PITG_07550*), *Avrvnt1* (*PITG_16294*) and one recently described *Avr-1* like gene (*PITG_06432*) were found in all of the isolates of *P. infestans*, *P. ipomoeae*, and *P. mirabilis*. The *Avr1* gene (*PITG_16663*) was missing from most isolates of *P. infestans,* but it was present in the isolates of clonal lineages US-1 and US-22. This gene was also not detected in the isolates of *P. mirabilis*, but was detected in the isolates of *P. ipomoeae*. The *Avr2* gene was found in all samples, except 3 isolates of *P. mirabilis*. The *Avr3a* gene (*PITG_14371)* which is an important pathogenicity determinant in *P. infestans* (Bos et al. 2010) was present in all the isolates of *P. infestans,* regardless of their geographic origin. However, none of the isolates of *P. ipomoeae* and *P. mirabilis* had the *Avr3a* gene. The *Avr4* gene (*PITG_07387)* has been shown to have frameshift mutations resulting in truncated proteins which are not recognized by the corresponding *R*-gene (*R4* resistance) (van Poppel et al. 2008). The value of BOC for the *Avr4* gene was lower than or close to 0.90 in all the isolates of *P. ipomoeae*, *P. mirabilis* and *P.*

infestans from Mexico, including isolate blue13. The *Avr4* gene was absent in one of the isolates belonging to the US-22 lineage (IN20009T1) and one of the Mexican isolates of *P. infestans* (Pic97335/inf3). The *Avr-blb1* gene (*PITG_21388)* was present in all the isolates of *P. infestans* and one isolate of *P. mirabilis*. However, the *Avr-blb2* gene (*PITG_20300)* had an unusual pattern of presence/absence polymorphism. The *Avr-blb2* gene was present in all the *P. mirabilis* isolates, but it was absent in all of the isolates of *P. ipomoeae*. In *P. infestans*, *Avr-blb2* had BOC value lower than 0.90 for the isolates belonging to US-8 (1/2), US-22 (3/3), US-24 (1/1) and Mexico (3/5). Together, the results indicate that a large proportion of Avr genes across the populations show presence/absence variation, suggesting that Avr genes are isolate or species-specific.

2.4.4 Nucleotide diversity is higher at RXLR genes compared to reference genes

Nucleotide diversity at the RXLR genes was compared to a set of core orthologous genes. No sequence variation was detected at two out of 220 core RXLR effectors. Therefore, nucleotide diversity was calculated for remaining 218 RXLR genes that were present in all the isolates of *P. infestans*, *P. ipomoeae* and *P. mirabilis*. The nucleotide diversity of the RXLR genes was compared to that of 218 randomly selected core orthologous genes. A Kruskal-Wallis one-way analysis of variance was used to determine whether nucleotide diversity was statistically different between these groups of genes. Across all three species, nucleotide diversity was significantly higher for the set of conserved RXLR genes compared to randomly selected core orthologous genes ($P < 0.001$) (Fig. 3a). Diversity was the lowest at both RXLR and core orthologous genes in the samples from *P. ipomoeae*. Nucleotide diversity was not significantly different between the set of conserved RXLR genes and those which showed presence/absence polymorphism (Fig. 3b).

2.4.5 Population genetic analyses

Standard population genetic methods were used to evaluate the history of natural selection operating at these RXLR genes. Using a BOC threshold value of 0.90, 52 genes were identified that had sufficient coverage for population genetic analyses (Fig. 2). We first evaluated the ratio of nonsynonymous substitutions per nonsynonymous

sites (π_a) to synonymous substitutions per synonymous sites (π_s). This ratio (π_a/π_s) can be used as an indicator of selective constraint within species. A ratio less than one indicates negative or purifying selection, a ratio of one is expected if the gene is evolving neutrally and a ratio greater than one implies positive or diversifying selection. Eleven genes (*PITG_04090*, *PITG_04266, PITG_06308*, *PITG_09837, PITG_12276*, *PITG_14371, PITG_14986*, *PITG_18683, PITG_20300, PITG_22978* and *PITG_22990*) surveyed had a ratio of π_a/π_s greater than unity (Fig. 4). This may indicate that some of the amino acid variants within these genes are favored by natural selection.

Well characterized Avr genes from *P. infestans* were further evaluated in their patterns of genetic diversity. Haplotype diversity for all Avr genes in *P. infestans* was greater than 0.5 except for *Avr2* (Table 3). The *Avr2* gene had only one nonsynonymous substitution (N31K) which resulted in two haplotypes and showed low haplotype diversity. Haplotype diversity was highest for *Avr-blb1* in *P. infestans* (0.87). The *Avr-blb1* and *Avr-blb2* genes had the greatest number of haplotypes (13) followed by *AvrSmira2* with 12 haplotypes (Table 3). The *AvrSmira1* gene also had the greatest number of haplotypes in *P. mirabilis* and *P. ipomoeae*.

2.4.6 A subset of RXLR genes encode a nuclear localization motif

To determine the potential contribution of the RXLR genes in gene regulation and cell death induction, the presence of a nuclear localization signal (NLS) was investigated. The presence of a NLS motif was predicted in the C-terminus of 54 RXLR genes (Supplemental table S2). Seventeen of the RXLR genes with a predicted NLS motif are reported to have Avr activities. These genes include the members of *Avr2* family (*PITG_06071, PITG_06077, PITG_07499, PITG_08278, PITG_19617, PITG_20025*) and the members of *Avr-blb2* family (*PITG_04081, PITG_04085, PITG_04086, PITG_04090, PITG_04097, PITG_09632, PITG_15972, PITG_18683, PITG_20300, PITG_20301, PITG_20303*).

2.4.7 Robustness of protein secondary structure to genetic variation

To understand how the genetic variation present in these species is distributed across the genes and proteins and what functional consequences these variants may

have, the protein secondary structure was predicted. The genetic variation present in our samples was concentrated mainly in the predicted alpha helices of these RXLR proteins, rather than in the strands, coils or turns (Fig. 5a). Furthermore, higher polymorphism at nonsynonymous sites was present in the alpha helices (Fig. 5b). Likewise, mutations within the predicted alpha helical regions led to fewer structural disruptions than in other regions (Fig. 5b). For instance, amino acid substitutions at position 155 of *PITG_22798* did not alter/disrupt alpha helix conformation (Fig. 6). In a subset of genes, the genetic variation present in our sample is predicted to result in different secondary structural elements within the effector domain. This was the case for 3 Avr genes: *PITG_18683, PITG_07550*, *PITG_21388* and five NLS-RXLR genes: *PITG_12276, PITG_15038, PITG_15225, PITG_22798, PITG_23193* (Fig. 6).

2.4.8 Expression analysis

The pattern of gene expression was investigated for five RXLR genes: *PITG_12276*, *PITG_15038*, *PITG_19994*, *PITG_22978* and *PITG_22990*. All five genes had an NLS motif and three showed evidence of recent diversifying selection: *PITG_12276, PITG_22978* and *PITG_22990*. *PITG_15038* was chosen because natural variation in this gene resulted in many secondary structural differences among alleles. As a contrast, *PITG_19994,* a RXLR gene for which alleles did not differ in their predicted secondary structures, was chosen. The levels of gene expression were determined at six time points during infection using RT-PCR. The RXLR genes, *PITG_22798* and *PITG_15038,* were upregulated at early stages of infection (24hpi and 48hpi) in both *S. lycopersicum* and *S. pimpinellifolium* (Fig. 7). Furthermore, RT-PCR revealed that the RXLR genes *PITG_ 19994* and *PITG_12276* were also upregulated but only during the necrotrophic phase of infection, whereas *PITG_22990* was not induced in any stages of infection (Fig. 7).

2.5 Discussion

In this study, we conducted comprehensive comparative genomic analyses and investigated the evolutionary history, presence/absence polymorphism and genetic diversity of RXLR genes within a closely related group of *Phytophthora* species. We detected fewer RXLR genes in *P. ipomoeae* and *P. mirabilis* compared to *P. infestans*.

Many of the Avr genes (with the exception of *Avr1*) were present in *P. infestans* underscoring their importance in pathogenesis. A common pattern observed at effector genes across a range of pathogens is that these loci are enriched for amino acid variation within populations or species. In line with this observation, the RXLR genes, as a class, harbor greater genetic diversity compared to a set of core orthologous genes. Furthermore, we identified 11 RXLR genes that specifically showed signatures of diversifying selection.

Genetic variation is a fundamental prerequisite for a response to selection and therefore knowledge about the distribution and functional consequences of the standing genetic variation present in a pathogen population can be valuable for crop protection. One source of genetic variation is point mutations. Even a single point mutation can alter the structure of a protein which may result in loss of biological function (Alberts et al. 2002; Bhattacharjee and Biswas 2013). In this study, several RXLR genes showed changes in protein secondary structure caused by a single mutation. This is important because pathogenic mutations have been reported to change the secondary structure of the protein (Abrusán and Marsh 2016). Also, it is notable that most mutations occurred in the alpha helices, suggesting that this secondary structure element can tolerate more variation than other elements. Previous studies on the genetic variation across genomes of humans also showed that alpha helices are more robust to SNPs (Abrusán and Marsh 2016).

At a broader scale, the knowledge of the patterns of genetic variation across RXLR genes within pathogen species is valuable for identifying future targets for resistance breeding. Genes showing substantial allelic variation in the pathogen population may warrant lower priority as resistance breeding targets, since a rapid shift in allele frequency in the pathogen population following the introduction of a new resistant line would be nearly unavoidable. Instead, a strongly conserved RXLRs may be better targets for developing novel, more durable resistance.

The development and deployment of novel resistance specificities is a difficult task. One challenge facing breeders is the low durability new resistance specificities due to the rapid emergence of pathogens that overcome novel specificities. One common mechanism by which the "resistance-breaking" strains overcome new resistant

genes is through the loss of Avr genes (van Poppel et al. 2008; Bos et al. 2010; Gilroy et al. 2011). We observed that most modern lineages of *P. infestans* lack the *Avr1* gene, but it is present in the US-1 and US- 22 lineages. The loss of *Avr1* from *P. infestans* in the modern samples might be driven by the deployment of the *R1* gene (Yoshida et al. 2013).

In our study, we also observed that some Avr genes appeared to be truncated relative to the reference sequence. For example, the current study showed that BOC value for *Avr4* was less than 100 in most isolates, suggesting that the *Avr4* gene was truncated in some isolates. Early stop codons leading to truncated proteins have been reported as a mechanism by which *R4*-mediated resistance is overcome (van Poppel et al. 2008).

Another important Avr gene, *Avr3a,* was present in all the isolates of *P. infestans,* but absent in *P. ipomoeae* and *P. mirabilis*. This suggests that the *Avr3a* gene in *P. infestans* may be associated with pathogenesis of species of *Solanum*. Two alleles of *Avr3a* are present in *P. infestans*: $Avr3a^{K80I103}$ (KI allele) and $Avr3^{E80M103}$ (EM allele). The $Avr3a^{KI}$ form activates resistance in the presence of *R3a* gene, whereas the $Avr3a^{EM}$ does not. Heterozygous individuals or individuals homozygous for the EM allele are virulent on plants with *R3a* gene (Cooke et al. 2012). Isolates from the US-1 lineage and 1306 were homozygous for the KI allele, while the rest of the isolates were either homozygous for the EM allele or heterozygous. Natural selection for the loss of host recognition may favor the $Avr3a^{EM}$ allele in pathogen populations (Bos et al. 2006; Martin et al. 2013). Likewise, genetic variation at two AVR genes, *Avr-blb1* and *Avr-blb2*, was higher than at other Avr genes. This sequence variation may be associated with differences in virulence between strains.

RXLR effectors that have Avr activities have been reported to be translocated inside the plant cell (Schornack et al. 2009). These effectors can be directed to different compartments within the host (Kamoun 2006). Our study identified 54 RXLRs that are predicted to have NLS motifs in the C-terminus and are predicted to move to the nucleus, where they may manipulate host gene expression. For some effectors, the NLS is required for induction of hypersensitive cell death. For example, the NLS-RXLR effector *PITG_22279* has been reported to target the host nucleus and cause cell death

in an agroinfiltration assay in *Nicotiana benthamiana* (Wang et al. 2017). Interestingly, mutations disrupting the NLS region of *PITG_22279* prevented the protein to be localized to the nucleus and failed to trigger the cell death response (Wang et al. 2017).

Not only is the localization of an effector protein important for its function, but so is the timing of expression. Several RXLR effectors with Avr activities, including *Avr3a*, *Avr1d*, *Avr4* and *AVR-blb1* are expressed early during infection (Haas et al. 2009; W. Yin et al. 2013). This study showed that the RXLR effectors *PITG_15038* and *PITG_22798* were upregulated during the biotrophic phase of infection (24 hpi) by *P. infestans* in both cultivated and wild tomatoes. Similar to this study, Wang et al. (2017) found that the effector gene *PITG_22798* was expressed during early stages of potato infection. On the other hand, a few effector genes such as *PiNPP1.1* has been reported to be upregulated later during infection of tomato. Likewise, in the current study, two of the RXLR genes *PITG_12276* and *PITG_19994* showed expression at the necrotrophic phase of the infection in both *Solanum* species.

In conclusion, this study showed that the *P. infestans* isolates possess a large number of RXLR and Avr genes, suggesting their importance in host-pathogen interactions. Furthermore, 220 core RXLR effectors were conserved between three closely related species of *Phytophthora*. These core RXLR effectors can be used as targets in developing late blight resistant crops. This study also showed that diversifying selection has operated on 11 RXLR genes. Understanding the pattern of genetic diversity and evolutionary history gives us insights on how likely a pathogen can evolve novel infection strategies. This knowledge, in turn, can help to shape plant breeding programs for long-term crop protection.

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2.8 Tables

Table 1. The source of whole-genome sequence data used for the *P. infestans*, *P. ipomoeae* and *P. mirabilis* strains.

Species/Strain	Genotype	Accession no. [a]	Origin	Year	Reference
P. infestans					
1306_2	…	…	California	Unknown	Knaus et al. (2016)
blue13	…	ERS226850	England	2006	Cooke et al. (2012)
P13527	…	ERS226844	Ecuador	2002	Yoshida et al. (2013)
P13626	…	ERS226845	Ecuador	2003	Yoshida et al. (2013)
NL07434	…	ERS226846	The Netherlands	2007	Yoshida et al. (2013)
DDR7602	US-1	ERS226848	Germany	1976	Yoshida et al. (2013)
LBUS5	US-1	ERS226849	South Africa	2005	Yoshida et al. (2013)
RS2009P1	US-8	ERS258000	Pennsylvania	2009	Martin et al. (2013)
US040009	US-8	…	New York	Unknown	Knaus et al. (2016)
FP-GCC	US-11	…	New York	Unknown	Knaus et al. (2016)
P10127	US-18	ERS241587	North Carolina	2002	Yoshida et al. (2013)
IN2009T1	US-22	ERS258001	Pennsylvania	2009	Martin et al. (2013)
US10006	US-22	…	Kentucky		Knaus et al. (2016)

Species/Strain	Genotype	Accession no. [a]	Origin	Year	Reference
P. infestans					
P17777	US-22	ERS226847	New York	2009	Yoshida et al. (2013)
BL2009P4	US-23	ERS258002	Pennsylvania	2009	Martin et al. (2013)
FL2009P4	US-23	…	Kentucky	2009	Knaus et al. (2016)
ND822Pi	US-24	…	North Dakota	Unknown	Knaus et al. (2016)
Pic97146/inf2	…	…	Mexico	1997	Goss et al. (2014)
Pic97335/inf3	…	…	Mexico	1997	Goss et al. (2014)
Pic97750/inf5	…	…	Mexico	1997	Flier et al. (2002)
Pic97785/inf6	…	…	Mexico	1997	Goss et al. (2014)
P10650	…	ERS241584	Mexico	2004	Yoshida et al. (2013)
P. ipomoeae					
PIP-07-001/ipo1	…	…	Mexico	1999	Current study
PIP-07-003/ipo2	…	…	Mexico	1999	Current study
PIP-07-096/ipo4	…	…	Mexico	1999	Current study
PIP-07-097/ipo5	…	…	Mexico	1999	Current study
P. mirabilis					
PM-07-001/mir1	…	…	Mexico	1999	Current study

Species/Strain	Genotype	Accession no. [a]	Origin	Year	Reference
P. mirabilis					
PM-07-099/mir4	…	…	Mexico	1999	Current study
PM-07-100/mir5	…	…	Mexico	1999	Current study
P7722	…	ERS241588	Mexico	1992	Yoshida et al. (2013)

[a] Whole-genome sequencing was conducted for the strains with no accession numbers.

Table 2. The universal and gene-specific primers used in reverse transcription polymerase chain reaction (RT-PCR).

Primers	Sequence 5′–3′	Reference
COX2-F	GGCAAATGGGTTTTCAAGATCC	Hudspeth et al. (2000)
COX2-R	CCATGATTAATACCACAAATTTCACTAC	Hudspeth et al. (2000)
12276-F	ACTCTTTGCAAGCGCCAAAG	Current study
12276-R	ATCTGTGCGACGACCCTTTT	Current study
15038-F	TCTTCTGGCCAATCCGCAAT	Current study
15038-R	CAGTCTGCATCCTCTTGGCA	Current study
19994-F	GGTGCGGATATGGTCTCCAG	Current study
19994-R	TGTCGTCTGCTGCGTTAAGT	Current study
22798-F	AACAAGTTAGCTGCGGTCGA	Current study
22798-R	GAGCTCGGATCCAGACCTTG	Current study
22990-F	GAGAGCTGGCCAAGGACTTT	Current study
22990-R	CACCTTTGGGGATGTACGCT	Current study

Table 3. Number of well characterized avirulence genes, nucleotide haplotypes and haplotype diversity observed in *P. infestans*, *P. mirabilis*, and *P. ipomoeae*.

	***P. infestans* (22)**[a]			***P. mirabilis* (4)**			***P. ipomoeae* (4)**		
Avr gene	Isolates[b]	Haplotypes	HD[c]	Isolates	Haplotypes	HD	Isolates	Haplotypes	HD
Avr1	5	5	0.67	…[d]	…	…	4	4	0.78
Avr2	22	2	0.13	1	2	1	4	2	0.43
Avr3a	22	5	0.65	…	…	…	…	…	…
Avr4	21	8	0.69	…	…	…	…	…	…
Avr-blb1	22	13	0.87	1	2	1	…	…	…
Avr-blb2	17	13	0.69	4	1	0	…	…	…
AvrSmira1	22	5	0.73	4	8	1	4	8	1
AvrSmira2	22	12	0.57	3	5	0.93	…	…	…
Avrvnt1	22	8	0.67	4	4	0.86	4	4	0.78

[a] Sample size is indicated in parentheses.

[b] Number of isolates carrying the avirulence gene.

[c] Haplotype diversity.

[d] Genes inferred as missing using BOC of less than 0.90.

2.9 Figures

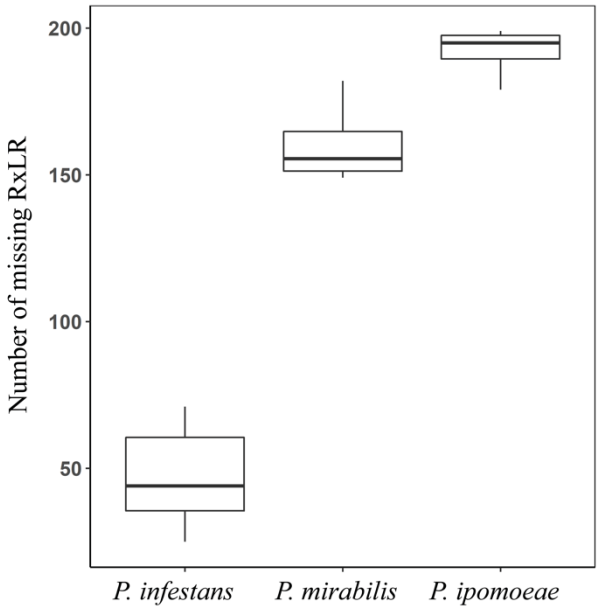


Fig. 1. Number of RxLR genes with no coverage in three *Phytophthora* species relative to *P. infestans* strain T30-4 (reference genome). Breadth of coverage (BOC) cutoff value was set at 0.90.

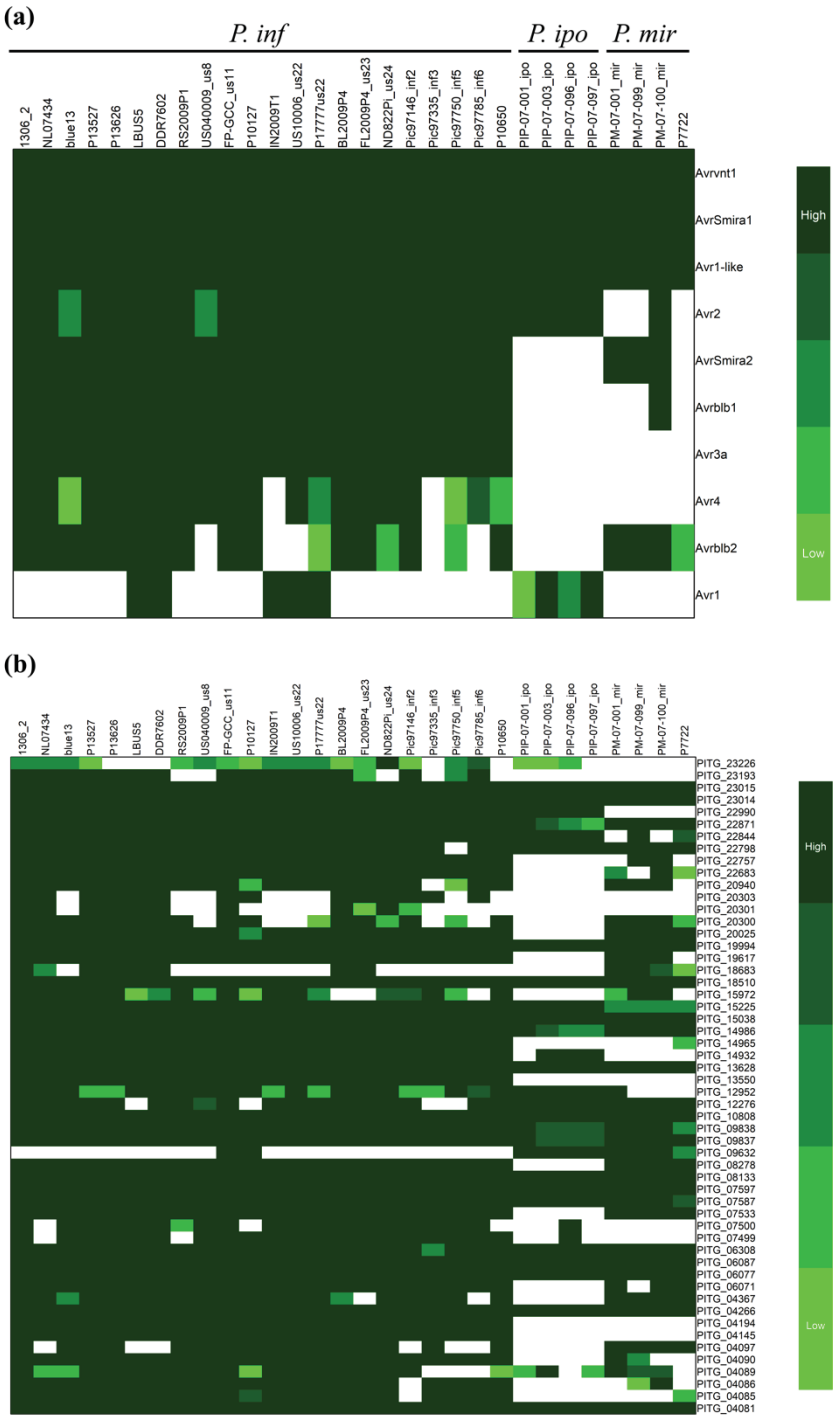


Fig. 2. Breadth of coverage for a) 10 avirulence and b) 54 NLS-RXLR genes in *Phytophthora* clade 1C genomes. The RXLR genes are indicated in rows and *Phytophthora* genomes are shown in columns. *Phytophthora infestans, P. ipomoeae* and *P. mirabilis* are indicated as *P. inf, P. ipo* and *P. mir*, respectively. Color indicates BOC value ranging from 0.90 (light green) to 1 (dark green). BOC value of less than 0.90 is indicated in white and, thus treated as missing.

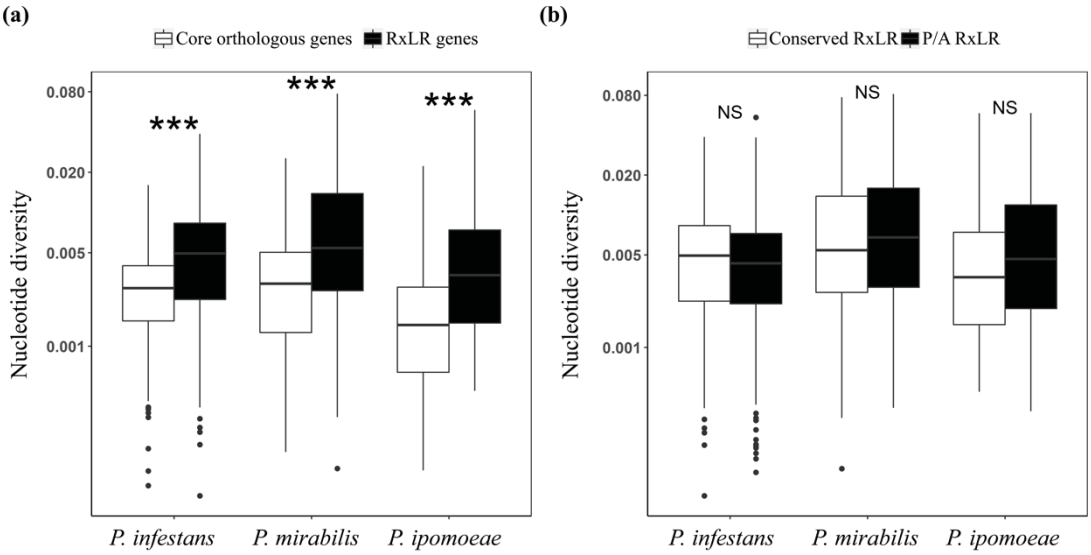


Fig. 3. Nucleotide diversity observed at core orthologous and RXLR genes in three *Phytophthora* species. **a)** Nucleotide diversity of 218 conserved RXLR genes compared with randomly selected 218 core orthologous genes for three *Phytophthora* species. **b)** Nucleotide diversity at conserved RXLR genes compared to RXLR genes with presence/absence polymorphism. Statistical differences were tested using Kruskal-Wallis test.

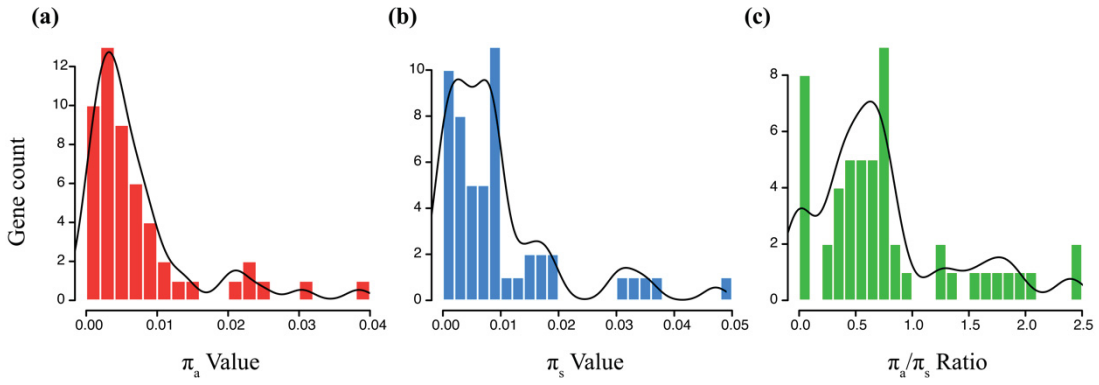


Fig. 4. Frequency distribution of sequence variation across 52 RXLR genes: **a)** nonsynonymous substitutions (π_a), **b)** synonymous substitutions (π_s) and **c)** the ratio (π_a/π_s) of nonsynonymous substitutions to synonymous substitutions for the RXLR genes. A ratio of π_a/π_s less than 1 indicates purifying selection ($\pi_a<\pi_s$), a ratio of 1 indicates neutral selection and a ratio above 1 indicates diversifying selection ($\pi_a>\pi_s$).

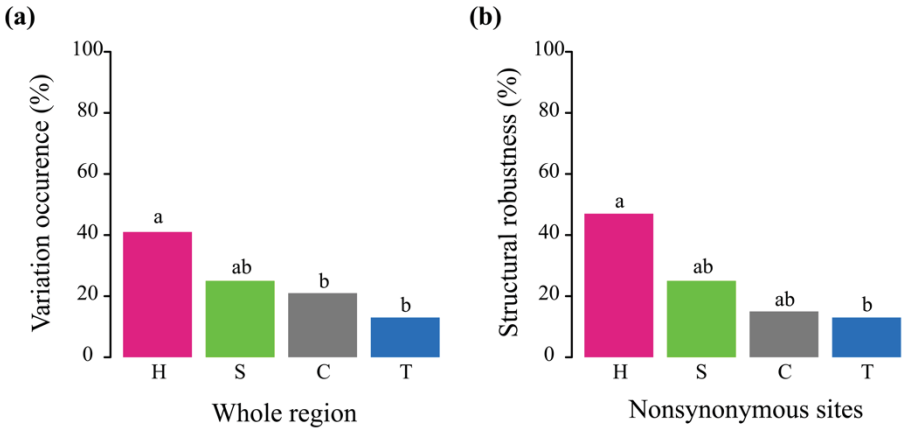


Fig. 5. The genetic variation present in the predicted secondary structural elements of RXLR proteins at **a**) the whole protein region and **b**) robustness of secondary structural elements at nonsynonymous sites. The alpha helices, beta strands, coils and turns are labeled H, S, C and T, respectively.

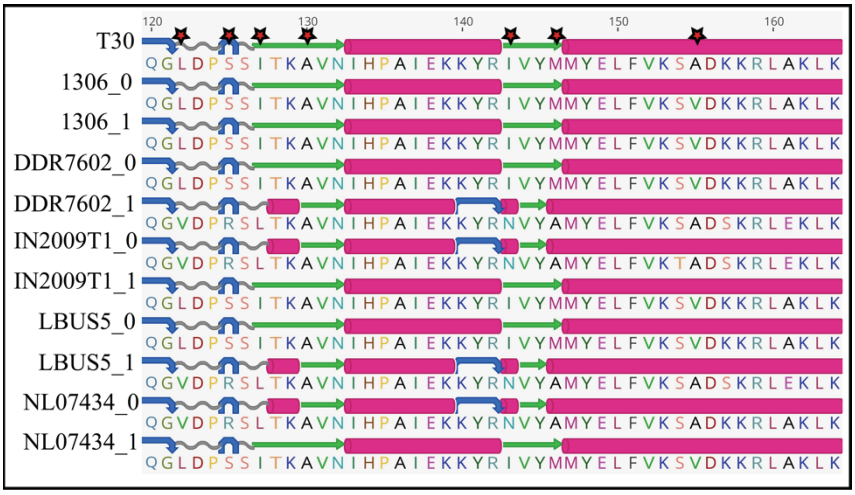


Fig. 6. Changes in predicted secondary structure of the NLS-RXLR gene *PITG_22798* caused by substitution mutations in the C-terminal domain. The location of mutations is indicated by a star. The reference genome for *Phytophthora infestans* is labeled T30. The two haplotypes for each isolate are labeled 0 and 1. Alpha helices, beta strands, coils and turns are indicated in deep pink, green, gray and blue, respectively.

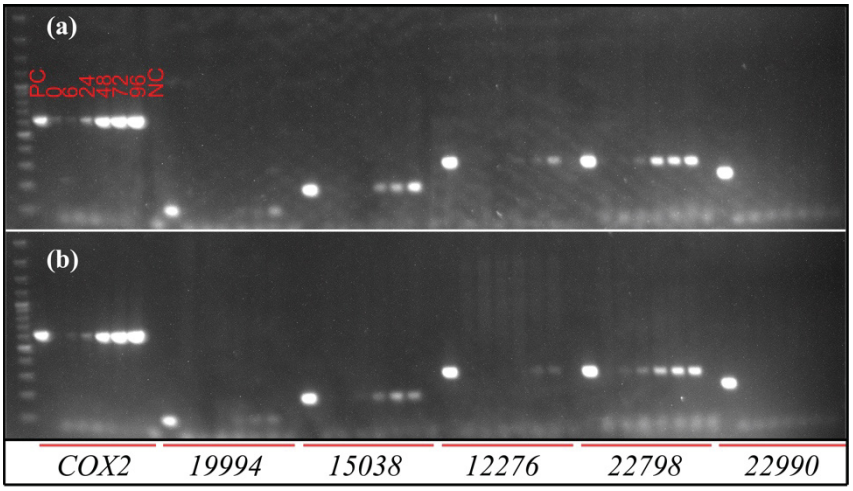


Fig. 7. Induction of NLS-RXLR genes during infection of *P. infestans* D12-2 on *Solanum* species. Reverse transcription polymerase chain reaction (RT-PCR) was conducted for five NLS-RXLR genes, as well as the *COX*2 region on **a)** *S. lycopersicum* and **b)** *S. pimpinellifolium.* The plants were inoculated with *P. infestans* D12-2 and samples were taken at six time points (0 hpi, 6hpi, 24 hpi, 48 hpi, 72 hpi and 96 hpi). Samples were loaded into the gel from left to right in the following order: positive control (PC), 0 hpi, 6 hpi, 24 hpi, 48 hpi, 72 hpi, 96 hpi and negative control (NC). A 100bp DNA ladder (L) was used for the size of the fragments on a gel, during electrophoresis.

2.10 Supplemental tables

Supplemental table S1. Presence/absence polymorphisms of 343 RXLR genes belonging to 106 gene families.

RXLR Family	PA genes	Total genes	Percent PA
RXLRfam1	42	85	0.49411765
RXLRfam5	21	22	0.95454546
RXLRfam9	16	17	0.94117647
RXLRfam7	15	18	0.83333333
RXLRfam6	13	18	0.72222222
RXLRfam21	10	11	0.90909091
RXLRfam8	9	10	0.9
RXLRfam2	8	17	0.47058824
RXLRfam17	7	8	0.875
RXLRfam3	7	11	0.63636364
RXLRfam32	6	8	0.75
RXLRfam4	6	8	0.75
RXLRfam10	5	11	0.45454546
RXLRfam18	5	8	0.625
RXLRfam20	5	9	0.55555556
RXLRfam38	5	6	0.83333333
RXLRfam39	5	6	0.83333333
RXLRfam43	5	6	0.83333333
RXLRfam50	5	5	1
RXLRfam42	4	6	0.66666667
RXLRfam51	4	5	0.8
RXLRfam54	4	5	0.8
RXLRfam58	4	5	0.8
RXLRfam69	4	4	1
RXLRfam13	3	3	1
RXLRfam14	3	4	0.75
RXLRfam23	3	7	0.42857143
RXLRfam25	3	5	0.6
RXLRfam37	3	7	0.42857143
RXLRfam55	3	4	0.75
RXLRfam67	3	4	0.75
RXLRfam84	3	3	1
RXLRfam86	3	3	1
RXLRfam93	3	3	1
RXLRfam95	3	3	1
RXLRfam98	3	3	1
RXLRfam109	2	2	1
RXLRfam121	2	2	1

RXLR Family	PA genes	Total genes	Percent PA
RXLRfam124	2	2	1
RXLRfam126	2	2	1
RXLRfam128	2	2	1
RXLRfam15	2	3	0.66666667
RXLRfam16	2	9	0.22222222
RXLRfam24	2	2	1
RXLRfam28	2	3	0.66666667
RXLRfam36	2	3	0.66666667
RXLRfam40	2	2	1
RXLRfam46	2	3	0.66666667
RXLRfam52	2	4	0.5
RXLRfam56	2	5	0.4
RXLRfam59	2	5	0.4
RXLRfam81	2	3	0.66666667
RXLRfam83	2	3	0.66666667
RXLRfam88	2	3	0.66666667
RXLRfam89	2	2	1
RXLRfam91	2	3	0.66666667
RXLRfam97	2	3	0.66666667
RXLRfam99	2	3	0.66666667
RXLRfam100	1	3	0.33333333
RXLRfam108	1	2	0.5
RXLRfam110	1	2	0.5
RXLRfam113	1	1	1
RXLRfam115	1	2	0.5
RXLRfam116	1	1	1
RXLRfam117	1	2	0.5
RXLRfam118	1	1	1
RXLRfam119	1	2	0.5
RXLRfam12	1	1	1
RXLRfam120	1	2	0.5
RXLRfam122	1	1	1
RXLRfam125	1	2	0.5
RXLRfam127	1	2	0.5
RXLRfam19	1	2	0.5
RXLRfam22	1	2	0.5
RXLRfam26	1	5	0.2
RXLRfam27	1	4	0.25
RXLRfam30	1	2	0.5
RXLRfam34	1	1	1
RXLRfam45	1	6	0.16666667
RXLRfam47	1	5	0.2
RXLRfam48	1	1	1

RXLR Family	PA genes	Total genes	Percent PA
RXLRfam53	1	2	0.5
RXLRfam57	1	2	0.5
RXLRfam66	1	2	0.5
RXLRfam70	1	2	0.5
RXLRfam71	1	1	1
RXLRfam82	1	2	0.5
RXLRfam85	1	3	0.33333333
RXLRfam96	1	1	1
RXLRsng157	1	1	1
RXLRsng162	1	1	1
RXLRsng163	1	1	1
RXLRsng164	1	1	1
RXLRsng167	1	1	1
RXLRsng184	1	1	1
RXLRsng187	1	1	1
RXLRsng192	1	1	1
RXLRsng203	1	1	1
RXLRsng204	1	1	1
RXLRsng209	1	1	1
RXLRsng217	1	1	1
RXLRsng221	1	1	1
RXLRsng237	1	1	1
RXLRsng241	1	1	1
RXLRsng242	1	1	1
RXLRsng247	1	1	1

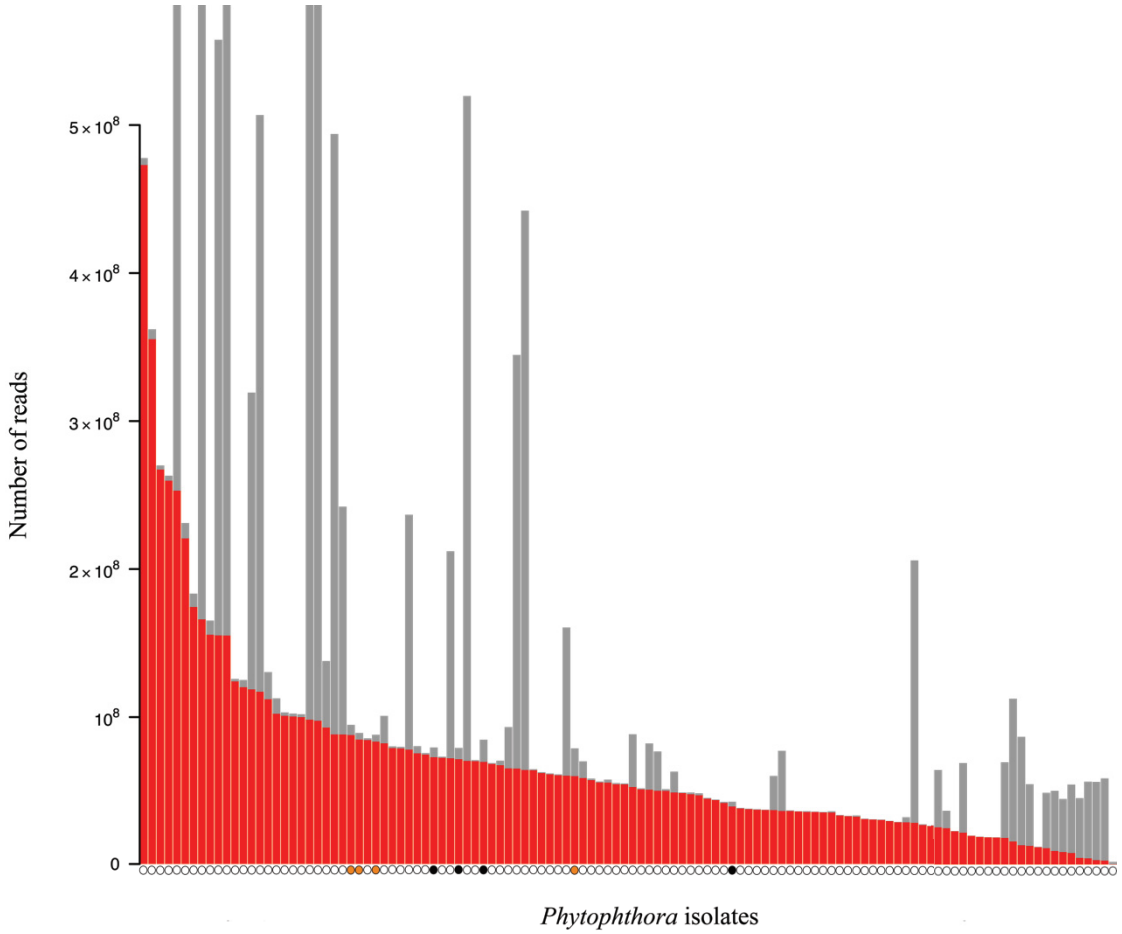
Supplemental table S2. Prediction of a nuclear localization signal (NLS) motif in RXLR genes using NLStradamus.

RXLR genes	Predicted NLS motif
PITG_15038	RLSKRRKTSPK
PITG_19994	RKPRARRSWPRAFKGNNSK
PITG_04145	LSKASAKKVKKAGKAVKEL
PITG_06308	GNKKTPNGMRRKIK
PITG_07587	KKEKRGKPRKD
PITG_22757	KLRANRLAEKLKSTATAKKLLATAKKTSSSLKKVPGVKSVQKRILYNRLSRWAKQGKRPEDLKKAGK
PITG_10808	RKRKLRARNTRK
PITG_13550	KQARETEKVKKAAKAKQKRLNKMRHAQGK
PITG_18510	LGKKA
PITG_13628	IFKIISRLKKLKLKR
PITG_06077	KKAKEAAKRKK
PITG_07499	KKAKEKAEKI
PITG_12276	IKKGRRTDPRSKNK
PITG_06087	KEEKK
PITG_22844	AEKLKNRKLRKL
PITG_08133	AKKLSGVRKQKKR
PITG_14986	KKNKKKK
PITG_22798	KKRLAKLKRKR
PITG_22990	PKVKKFKPSKP
PITG_23014	PKQKKLAAALKKSTSK
PITG_23015	PKQKKLAAALKKSTSK

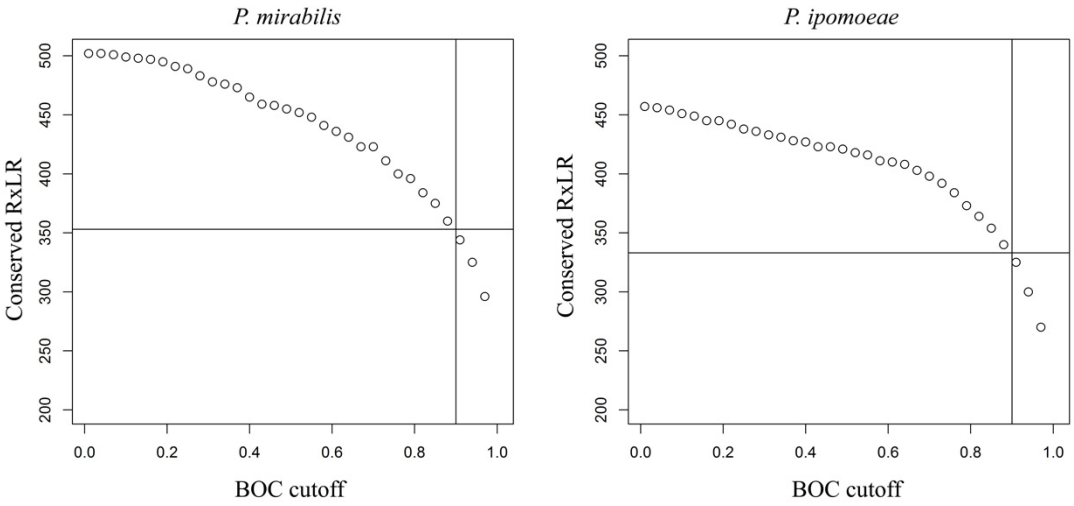
RXLR genes	Predicted NLS motif
PITG_23226	KPKHKKLAAALKKSTSKHK
PITG_07533	KRAAKKG
PITG_22871	RRGRKK
PITG_15225	KKFRDEAVRRQRKRNQAK
PITG_07597	KKGERKPFIEAKLKKALANPK
PITG_12952	RKKP
PITG_04367	TRGKLERSKAAKAR
PITG_20940	TRGKLERSKAAKARW
PITG_06071	RKIRERKK
PITG_07500	AKKAKEKAEKIK
PITG_08278	KKPNPAKAAKKAKEKAAKIK
PITG_19617	KKLNPAKAAKKAKEKAAKIK
PITG_20025	KKPNPAKAAKKAKEKAAKIK
PITG_09837	KKEKARSKA
PITG_09838	KKEKARSKA
PITG_14932	HKHLRRLKR
PITG_14965	HKHLRRLKR
PITG_04081	AAAKKAKLEKMKEK
PITG_04085	KKPDIKISKLIEAAKKAKKKMTKS
PITG_04086	KKPDIKISKLIEAAKKAKKKMTKS
PITG_04089	KLIAAAKKALLEKKMAKLSKVIKKPAK
PITG_04090	KISKLIAAAKKAKAKMTK

RXLR genes	Predicted NLS motif
PITG_04097	AKKAKKVKKLKNLMKLKKSS
PITG_04194	AKKAKDLKKIKNLISRKK
PITG_09632	KVKKLKE
PITG_18683	KKPDIKIGKLIEAAKKAKKKMTKS
PITG_20300	KIGKLIEAAKKAKAKMTK
PITG_20301	KKPDIKISKLIAAAKKAKAKMTKS
PITG_20303	KKPDIKISKLIAAAKKAKAKMTKS
PITG_23193	EKLKK
PITG_15972	PAKAAKKAK
PITG_22683	KPKVRRLSEVRARRDAHRSRKADQGPTGARR
PITG_04266	FKRRKRKKKKKKHK

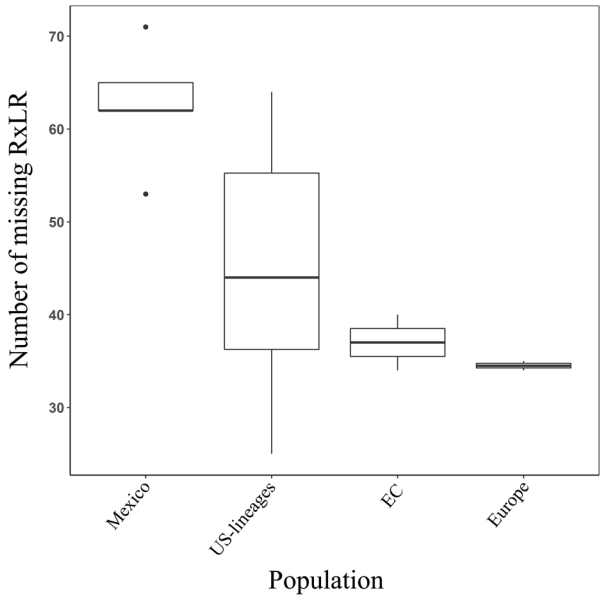
2.11 Supplemental figures



Supplemental fig. S1. Library size (reads sequenced) for samples. The isolates of *P. infestans*, *P. ipomoeae* and *P. mirabilis* are indicated as white, orange and black circles, respectively. Total read count is indicated in gray and number of mapped reads is indicated in red.



Supplemental fig. S2. Predicted number of conserved RxLR genes in *P. mirabilis* and *P. ipomoeae* with increasing BOC cutoff value. At BOC value of 0.90, approximately 350 RxLR genes were conserved.



Supplemental fig. S3. Number of RXLR genes with no coverage in sexual and clonal populations of *P. infestans*. The Mexican population *P. infestans* have fewer RXLR genes compared to other populations.

CHAPTER 3

EFFICACY OF BIOLOGICAL AGENTS AND COMPOST ON GROWTH AND RESISTANCE OF TOMATOES TO LATE BLIGHT

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3.1 Abstract

The management of plant diseases are dependent on a variety of factors. Two important variables are the soil quality and its bacterial/fungal community. However, the interaction of these factors is not well understood and remains problematic in producing healthy crops. Here, the effect of oak–bark compost, *Bacillus subtilis* subsp. *subtilis*, *Trichoderma harzianum* and two commercial products (FZB24 and FZB42) were investigated on tomato growth, production of metabolites and resistance under biotic stress condition (infection with *Phytophthora infestans*). Oak–bark compost, *B. subtilis* subsp. *subtilis*, and *T. harzianum* significantly enhanced plant growth and immunity when exposed to *P. infestans*. However, the commercial products were not as effective in promoting growth, with FZB42 having the weakest protection. Furthermore, elevated levels of anthocyanins did not correlate with enhanced plant resistance. Overall, the most effective and consistent plant protection was obtained when *B. subtilis* subsp. *subtilis* was combined with oak–bark compost. In contrast, the combination of *T. harzianum* and oak–bark compost resulted in increased disease severity. The use of compost in combination with bio-agents should, therefore, be evaluated carefully for a reliable and consistent tomato protection.

3.2 Introduction

Cultivated tomato (*Solanum lycopersicum* L.) has a global production of 3.7 million hectares and is one of the most valuable agricultural crops worldwide (fao.org). However, tomatoes and nearly every crop species are also susceptible to a variety of pathogens that reduce both yield and quality. Late blight is the most devastating disease worldwide of both tomato and potato (Agrios 2005). Annual crop losses from late blight are estimated at over five billion USD (Judelson and Blanco 2005; Haverkort et al. 2009). Late blight is caused by *Phytophthora infestans* (Mont.) de Bary. This pathogen belongs to the Oomycota, a distinct lineage of filamentous eukaryotes which are fungus-like. This pathogen infects multiple plant species in the Solanaceae, including potato and tomato (Fry et al. 2015). In the mid-19th century, *P. infestans* devastated the potato crop and caused the Irish potato famine (Fry et al. 2015). To this day, disease management remains difficult and requires integrated management strategies.

Late blight affects the leaves, stems, and fruits of tomato and can cause total crop loss within as little as 2 weeks. The most effective control of late blight is by chemical fungicides. Phenylamide fungicides, such as mefenoxam, have been used against *P. infestans* and provide an effective disease suppression (Saville et al. 2015). However, chemical applications have detrimental outcomes to environmental and human health (Schummer et al. 2012a, b). Another major problem with the use of fungicides is the evolution of fungicide resistance in *P. infestans* populations (Taylor et al. 2002; Matson et al. 2015). Therefore, fungicides cannot be used continuously and do not represent a sustainable method of control. To maintain crop yields, without damaging the environment and human health, organic amendments and biocontrol agents have been explored as alternatives to chemical fungicides.

In horticulture, the application of compost to soil improves soil structure and plant root growth and results in an overall increase in yield of several crops, such as tomato (Gutierrez-Miceli et al. 2007). Furthermore, compost provides essential plant nutrients such as nitrogen, phosphorus, and calcium and thereby reduces the need for synthetic fertilizers (Lewis et al. 1992; Iqbal et al. 2010). Composts can be made of raw feedstocks such as yard trimmings, food waste, manure, tree leaves/bark and worm castings (Termorshuizen et al. 2006). The antagonistic and biological effect of compost for disease suppression is quite well known. Compost has been used as an organic treatment for disease suppression against many soilborne pathogens, including oomycete species, *Rhizoctonia solani* Kühn and *Fusarium* species in tomato, cauliflower, rooibos, oats, lupin, pine, and flax (Termorshuizen et al. 2006; Bahramisharif et al. 2013; Tewoldemedhin et al. 2015; Lamprecht and Tewoldemedhin 2017). Composts, such as non-aerated compost teas, have also shown significant suppressive effects on foliar pathogens, where mycelial growth of *P. infestans* was completely inhibited *in vitro* (Kone et al. 2010). However, information is limited on the use of compost to control *P. infestans* in greenhouse or field conditions. Various factors may influence disease suppression by composts and thus, the effect of compost is not always consistent. These factors include compost composition, microbial biomass, the rate of application and maturity (Termorshuizen et al. 2006; Janvier et al. 2007). On the other hand, the application of some composts may be problematic, especially those rich in saline, which have been shown to enhance oomycete disease severity (Hoitink et al.

1997). Therefore, the use of composts and their nutrient content must be carefully evaluated to achieve consistent plant growth and disease suppression.

Biological agents (bio-agents), defined as living organisms, can significantly lower the density of plant pathogens (O'Brien 2017). Biological control has therefore become very popular as a non-chemical alternative to control late blight disease. In the last three decades, numerous bacterial bio-agents have been evaluated for their ability to suppress *P. infestans.* These include: *Bacillus amyloliquefaciens* Priest et al*.,* *Bacillus cereus* Frankland and Frankland, *Bacillus pumilus* Meyer and Gottheil, *Bacillus subtilis* Ehrenberg*,* and *Pseudomonas fluorescens* Migula (Yan et al. 2002; An et al. 2010; Chowdappa et al. 2013). *Bacillus* species have shown great potential to promote plant growth and suppress late blight in tomato. Kabir et al. (2013) evaluated 125 different soil microbes and described six strains of *Bacillus* that suppressed late blight by more than 60% on culture plates and *in planta*. They also showed that these bio-agents are able to enhance plant growth. Some of these bio-agents are known as plant growth promoting rhizobacteria (PGPRs). Two of the most effective PGPRs are *B. subtilis* and *B. amyloliquefaciens*. They are both Gram-positive bacteria, commonly found in soil. The PGPRs colonize the root and promote plant growth and most importantly enhance protection against plant pathogens. Furthermore, *B. subtilis* and *B. amyloliquefaciens* have been shown to stimulate the plant immune system by activating plant induced systemic resistance (ISR) and promote growth in several crops, including tomato (Kloepper et al. 2004; Chowdappa et al. 2013). *Bacillus subtilis* and *B. amyloliquefaciens* are both available as commercial products, marketed as Serenade® (*B. subtilis,* strain QST 713), FZB24®WG (*B. amyloliquefaciens,* strain FZB24) and RhizoVital® 42 (*B. amyloliquefaciens,* strain FZB42).

There are numerous fungal antagonists that are also available as potential biocontrol agents. The fungal agents that are capable of suppressing *P. infestans* include *Fusarium oxysporum* Schlecht. emend. Snyder and Hansen*, Pythium oligandrum* Dreschler and *Trichoderma* species (Kim et al. 2007; Horner et al. 2012; Yao et al. 2016). *Trichoderma harzianum* Rafai and *P. oligandrum* have been reported to suppress the pathogen through competition, promoting plant growth and antibiosis or through mycoparasitism (Benhamou et al. 1999; Benitez et al. 2004). Similar to PGPRs, *Trichoderma* species stimulate plant immunity which may result in an activation of ISR

in plants. Several studies have reported that *T. harzianum* upregulated induced defense response in different plants, including maize and tomato (Martinez-Medina et al. 2013; Saravanakumar et al. 2016).

While a few studies have shown that the use of organic amendments such as compost in combination with non-pathogenic species or biocontrol agents could significantly improve the disease suppression caused by highly virulent soilborne pathogens (Hoitink and Boehm 1999; Bahramisharif et al. 2013), the complex and inconsistent management of biological control has not translated into widespread use in field crops (Ryan et al. 2004; Xu et al. 2011). In particular, the potential of a combination compost and biological control treatment in tomato protection has not been fully exploited. The aim of this study was to identify reliable methods for improving plant resistance under biotic stress in tomato production. We evaluated (1) the effect of biological agents and commercial products on tomato growth, stress response and protection, (2) the potential of oak–bark compost as a standalone treatment or in combination with bio-agents in plant growth, stress and protection, and (3) whether a combination of the compost with the biological agents or the commercial products increases consistency.

3.3 Materials and methods

3.3.1 Biological agents

In this study, two biocontrol agents were tested for the ability to control late blight disease in tomato: *Bacillus subtilis* subsp. *subtilis* and *Trichoderma harzianum*. The *Bacillus subtilis* subsp. *subtilis* isolate DSM-10 was sourced from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). The *T. harzianum* isolate CBS 354.33 was obtained from the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). Furthermore, two commercial products containing *B. amyloliquefaciens* ssp. *plantarum*: FZB24®WG and RhizoVital® 42/FZB42 were used. Both products were purchased from ABiTEP—Biotech for Agriculture and Ecology (Berlin, Germany).

3.3.2 Isolation and sporulation of *Trichoderma harzianum*

The *T. harzianum* isolate CBS 354.33 was grown on Trichoderma-selective media (Williams et al. 2003). The culture was incubated at 24 °C for 30 days in darkness. For sporulation of the isolate, the culture was plated out onto potato-dextrose agar (PDA) and incubated at 24 °C for 20 days. Ten ml ddH_2O was added to each plate and the plates were carefully sealed and incubated at room temperature for up to 2 h. The spores were then harvested as previously described (Perelló et al. 2009). The spore concentration was measured with a hemacytometer and a suspension with a concentration of 5×10^8 spores per ml was prepared and used immediately.

3.3.3 Isolation and sporulation of *Bacillus* spp.

Bacillus subtilis subsp. *subtilis* isolate DSM-10 was received as a freeze-dried culture. Following the supplier's instructions, the dried pellet was rehydrated with 0.5 ml of nutrient broth. After 30 min of incubation at room temperature, the content was gently mixed and about half of the content was transferred into a 5 ml tube containing nutrient broth. The other half was streaked onto nutrient agar plates and used for storage. The broth cultures were incubated on rotary shaker at 200 rpm at 30 °C (Nakamura et al. 1999) until the logarithmic phase was reached. The OD_{600} value was calculated using a DeNovix DS-11 FX spectrophotometer (DeNovix Inc., Wilmington, DE, USA). The suspension was diluted to reach the concentration of 5×10^8 colony forming units per ml (CFU/ml).

The two commercial products, FZB24®WG and RhizoVital® 42/FZB42, contain living spores of *B. amyloliquefaciens* ssp. *plantarum*. These products were first diluted in ddH2O and 100 µl of the suspension was streaked on Luria–Bertani broth (LB) medium containing 1.5% agar. The cultures were incubated at 30 °C for 2 days and the bacterial cells were then harvested and grown in LB. The broth cultures were incubated at 30 °C overnight on rotary shaker (at 200 rpm). The spore concentration of 5×10^8 CFU/ml was prepared exactly as for DSM-10 and used immediately.

3.3.4 Biomass and plant height

To evaluate the effect of the treatments on plant growth, 3-week-old tomato plants were first carefully removed from the pots. The soil adhering to the roots was removed by gentle shaking and the remaining rhizosphere soil was brushed off into a 50 ml falcon tube for DNA extraction. The root was washed with water thoroughly to remove soil particles adhering to the root. Root and shoot length, as well as, fresh weight was then determined for all plants.

3.3.5 Efficacy of biocontrol

Twelve different treatments were evaluated for their ability to enhance plant growth and/or to suppress disease (Table 1). Each treatment had nine biological replicates, of which three were used to evaluate growth and six were used to evaluate disease suppression. The experiment was replicated two times. The replicates were carried out in a growth chamber under standard growing conditions for tomatoes. The replicates followed a randomized block design with the placement of the pots being changed every 7 days.

Five treatments contained un-amended soil (*Stender*® C-400 with Cocopeat) and seven treatments contained the same soil amended with 25% v/v of oak–bark compost. For all treatments, 11-cm diameter plastic pots were filled with approximately 1 kg of either soil or the soil amended with compost. One hole with a depth of 1.5 cm was made in the soil using 1 cm diameter sterile doweling rods. Directly before planting the tomato seed, 1 ml of biocontrol agent suspension was pipetted into the hole according to the treatment. Combined treatments received 1 ml suspension for all agents. Directly afterwards, one tomato seed (cv. Moneymaker) was planted into the hole and covered.

3.3.6 Isolation and sporulation of *P. infestans*

The highly virulent *P. infestans* isolate, D 12-2, was obtained from Francine Govers' Laboratory of Phytopathology (Wageningen University, The Netherlands). This isolate was grown on Rye B Agar (Caten and Jinks 1968). The medium was

prepared with 60 g of rye grain soaked in ddH_2O for 24 h. The supernatant was then removed and 1 l ddH_2O was added. The mixture was then boiled for 2 h in a 2 l sterile beaker. The supernatant was filtered through cheesecloth and combined with the original supernatant. Then, 15 g of Bacto agar, 20 g of sucrose and 0.05 g beta-sitosterol were added to the supernatant and autoclaved at 15 psi for 20 min. Following incubation on plates at 18 °C for 20 days in darkness, 10 ml of cold ddH_2O was used to harvest *P. infestans* sporangia. A zoospore suspension was then prepared by placing the suspension at 4 °C for 2–4 h until the zoospores were released (de Vries et al. 2017). A suspension with a total concentration of 5×10^5 zoospores/ml was prepared for infection using a hemacytometer.

3.3.7 Plant biotic stress assays (whole plant and detached leaf infection assays)

Whole plant infections were done on 3-week-old tomato plants using artificial inoculation technique as follows: Ten µl of the *P. infestans* zoospore suspension (5×10^5 zoospores/ml) was carefully infiltrated into the extracellular space of five young leaves using a 1 ml needleless syringe. For the control plants, 10 µl of ddH_2O was infiltrated into five leaves using the same technique. The inoculated leaflets were collected after 5 days. Re-isolation was made from the leaflet and root fulfilling Koch's postulates.

In detached leaf infection assay, five young leaves from 3-week-old plants were excised and placed in a Petri dish, containing wet sterile paper towel. Ten µl of the zoospore suspension (5×10^5 zoospores/ml) was loaded onto the abaxial surface of detached leaves. For the control plants, 10 µl of ddH_2O was loaded. The Petri dishes were kept at 18 °C in the dark for 5 days.

3.3.8 Screening of necrotic lesions by *P. infestans*

For both the whole plant and detached leaf assays, all inoculated leaves were bleached using 100% EtOH for 72 h. After bleaching out the chlorophyll, the necrotic lesions were examined under a SteREO Discovery V8 binocular (AxioCam ICc 5 camera; Zeiss, Jena, Germany) and quantified with the ZEN lite 2012 software (Zeiss).

3.3.9 Defense-related compounds

Anthocyanins, phenolic flavonoid pigments, are synthesized by the phenylpropanoid pathway and may be induced in response to plant stress. To determine whether the compost or the bio-agents affected the level of anthocyanin production in the leaves, the anthocyanin content was evaluated as previously described (Lindoo and Caldwell 1978). Six biological replicates were used for each treatment.

3.3.10 Design of specific primers for *T. harzianum* and *B. subtilis* subsp. *subtilis*

The ITS sequence of *T. harzianum* (CBS 354.33; AF278790) and 16S rRNA sequences of *B. subtilis* subsp. *subtilis* (DSM-10; LN681568), *B. amyloliquefaciens* strains FZB24 (AY055219) and FZB42 (AY055221) were downloaded from GenBank (https://www.ncbi.nlm.nih.gov) and uploaded into Geneious® 11.0.2 (Kearse et al. 2012). Species-specific primers were designed for all sequences using the Geneious plugin Primer 3 2.3.7 (Rozen and Skaletsky 1999) (Table 2).

3.3.11 DNA extraction and PCR amplification of *P. infestans* and *T. harzianum* isolates

For positive controls of the PCR amplification, *Phytophthora infestans* was grown on 20% unclarified V8 Agar (200 ml V8 juice, 800 ml ddH_2O, 15 g agar, 2 g $CaCO_3$ and 0.05 g beta-sitosterol) for 14 days and *T. harzianum* was grown on PDA for 7 days. Mycelium from both were then harvested and genomic DNA (gDNA) was extracted using the extraction method described by Edwards et al. (1991).

For DNA amplification of *P. infestans*, the cytochrome *c* oxidase subunit II (*COX2*) region was used (Hudspeth et al. 2000; Table 2). For *T. harzianum,* DNA was amplified for the specific primers described above. Polymerase chain reaction (PCR) was conducted in a total volume of 20 µl consisting of Green GoTaq® Flexi Buffer, 2 U GoTaq® Flexi DNA Polymerase (Promega, Fitchburg, WI, USA), 1.25 mM $MgCl_2$, 0.1 mM dNTPs and 0.2 mM of each primer. The T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) was used for amplification of *P. infestans* with PCR condition as follows: initial denaturation at 95 °C for 3 min, 34 cycles of 95 °C for 30 s, annealing

for 30 s at 60 °C, extension at 72 °C for 1.30 min, and final extension at 72 °C for 5 min. The PCR for *T. harzianum* was carried out with initial denaturation at 94 °C for 5 min, 32 cycles of 94 °C for 30 s, annealing for 30 s at 55 °C, extension at 72 °C for 1.30 min, and final extension at 72 °C for 7 min. Gel electrophoresis was analyzed by resolving PCR products in 1% agarose gel and DNA was stained using Midori Green Advance (Nippon Genetics Europe, Dueren, Germany).

3.3.12 DNA extraction and PCR amplification from bacterial isolates

The bacterial cells of DSM-10 were harvested from nutrient agar and then added into nutrient broth. For FZB24 and FZB42, the cells were harvested from LB medium. The broth media were incubated at 30°C for overnight on rotary shaker at 200 rpm. Chromosomal DNA was then extracted using DNeasy® PowerLyzer® Microbial Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. For the amplification of 16S rDNA, the universal primers V5F and V6R (Arenz et al. 2015) and newly designed primers were used (Table 2). PCR was carried out in the T100™ Thermal Cycler (Bio-Rad) with initial denaturation at 94 °C for 9 min, following 30 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 45 s, and the final extension for 7 min at 72 °C. To conduct gel electrophoresis, PCR products were resolved in 1% agarose gel and DNA was visualized by staining with Midori Green Advance (Nippon Genetics Europe).

3.3.13 DNA extraction from rhizosphere soil and plant material

To evaluate relative abundance of different species in rhizosphere soil and plant material, gDNA was extracted from rhizosphere soil using DNeasy® PowerSoil® Kit (Qiagen), and roots and leaves using DNeasy® PowerPlant® Pro Kit (Qiagen) according to manufacturer's instructions. PCR was conducted for screening for the presence of *P. infestans* and the bio-agents using the same PCR conditions as described above.

3.3.14 Cloning and sequencing

To investigate the microbial community in oak–bark compost, gDNA was extracted from the compost using DNeasy® PowerSoil® Kit (Qiagen). PCR was

conducted for amplification of DNA for the ITS and 16S rDNA regions with the same conditions as described above. The PCR product of compost's DNA was purified using peqGOLD Cycle-Pure kit (VWR, Peqlab, Radnor, PA, USA). The purified PCR product was then cloned using TOPO® TA cloning® kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. To screen the microbial community, sequencing analyses of the ITS and 16S rDNA regions of three clones were carried out by Eurofins Genomics DNA sequencing facility (Eurofins Genomics, Ebersberg, Germany). For the ITS region, the universal primers ITS6 (Cooke et al. 2000) and ITS4 (White et al. 1990) were used, and 16S rDNA region was amplified using primers V5F and V6R (Table 2).

3.3.15 Statistical analyses

To test for statistical differences between treatments, one-way ANOVA was performed for all the phenotypic measurements, including shoot and root length, plant fresh weight and necrotic area. Levene's test was conducted for homogeneity of two repeat trials (Levene 1961). Independent-samples *t* test was performed to compare the variances of all pairwise combinations. The Shapiro–Wilk's test was conducted to test for deviations from normality (Shapiro and Wilk 1965). Tukey's HSD (Honest significance different) test was calculated for each of these phenotypic measurements as well as anthocyanin content to determine significant differences at 5% level (Tukey 1949). All the procedures were performed by IBM SPSS Statistics software (version 25).

3.4 Results

3.4.1 Growth promotion by oak–bark compost and bio-agents

The null hypothesis of the Levene's test could not be rejected ($P > 0.05$); therefore, the data from the two repeat trials were combined. The Shapiro–Wilk's test showed that growth data do not fit the normal distribution. Thus, before the analysis of variance, the data were transformed to meet the assumption of normality using logarithmic transformation. According to the one-way analysis of variance plant growth differed significantly across treatments (Table 3).

Treatments with oak–bark compost and certain bio-agents significantly increased tomato growth (Figs. 1, 2). The compost-treated plants (T1) were the largest. Compared to the control plants (T0), plants grown with compost (T1) had 3.7-fold longer roots, 1.4-fold longer shoots and 3.3-fold greater fresh weight (Figs. 1, 2a–e). The two bio-agents, *B. subtilis* subsp. *subtilis* (T2) and *T. harzianum* (T8), significantly enhanced plant growth compared to the control (T0) (Figs. 1, 2a, b, e). The biomass was, however, larger by *B. subtilis* subsp. *subtilis* (T2) than *T. harzianum* (T3). The FZB24 product (T4) was better in promoting plant growth than FZB42 (T6). Treatment with FZB24 (T4) significantly improved root growth and fresh weight, while treatment with FZB42 (T6) did not significantly promote plant growth compared to the untreated control (T0) (Figs. 1, 2c–e).

3.4.2 Growth promotion by combination treatments

The assays showed that the combination of the compost and bio-agents or commercial products can further increase plant growth (Figs. 1, 2). *Bacillus subtilis* subsp. *subtilis* in combination with compost (T3) enhanced plant growth, compared to the stand-alone treatment with *B. subtilis* subsp. *subtilis* (T2) (Figs. 1, 2a). Compared to the control plants (T0), plants grown with a combination of oak–bark compost and *B. subtilis* subsp. *subtilis* (T3) had 3.8-fold longer roots, 1.6-fold longer shoots and 3.5-fold greater fresh weight (Figs. 1, 2a, e). Plants treated with FZB24 and compost (T5) had significantly longer shoots compared to the untreated control (T0) and had 1.2-fold longer shoots than in the standalone treatment with FZB24 (T4) (Figs. 1, 2c). Although FZB42 (T6) did not significantly stimulate plant growth compared to the untreated control, shoot length and fresh weight were significantly enhanced, when this commercial product was combined with the compost (T7) (Figs. 1, 2d).

3.4.3 Plant protection by oak–bark compost and bio-agents in whole plant assay

Based on Levene's test, variance from the two repeat trials were comparable and, thus data were combined. The normality test by Shapiro–Wilk was rejected for the necrotic area data and therefore, the data was transformed to meet the assumption of

normality using logarithmic transformation. Strong evidence for significant interactions was observed across treatments (Table 1).

All of the treatments evaluated in this study (T1–T11), except treatment T9, protected tomato plants from the disease (Fig. 3), while the untreated control plants remained highly susceptible to *P. infestans* (Figs. 4a, c). Oak–bark compost, as a stand-alone treatment (T1), suppressed the disease by 82% on average. The highest suppression of late blight (85%) was achieved in the treatment with *B. subtilis* subsp. *subtilis* (T2), but *T. harzianum* (T8) and FZB24 (T4) also showed high disease suppression (80% and 79%, respectively). In contrast, FZB42 (T6) was the least effective of all treatments, averaging 70% disease suppression.

3.4.4 Plant protection by co-inoculation of oak–bark compost and bio-agents in whole plant assay

The biological agents, in combination with the compost, improved plant protection, but not for all of the treatments (Fig. 3). Late blight disease severity decreased, on average, by 8% when the compost was combined with *B. subtilis* subsp. *subtilis* (T3) (Figs. 4b, e). Furthermore, the variance was significantly lower in combination treatments of compost with *B. subtilis* subsp. *subtilis* (T3), indicating consistent disease control. However, the combination of *T. harzianum* with compost (T9) resulted in a 23% increase in disease severity compared to *T. harzianum* alone (T8). The addition of *B. subtilis* subsp. *subtilis* to the treatment including *T. harzianum* and compost (T10) increased the suppression by 16% relative to T9. Furthermore, the negative impact of the combination of compost and *T. harzianum* was nullified when the bio-agents and commercial products were combined (T11), a treatment which resulted in an average total suppression of 80%, compared to the untreated control (T0).

3.4.5 Plant protection in detached leaf assay

In the detached leaf assay, oak–bark compost (T1) suppressed late blight disease by 71% and by *B. subtilis* subsp. *subtilis* (T2) suppressed late blight disease by 76% compared to the untreated control plants (T0) (Fig. 3). Furthermore, in the combined treatments, only treatments T3 and T10 reduced of late blight incidence compared to

the untreated control (T0) (Figs. 3, 4f). Similar to the whole plant assays, the disease severity was higher when *T. harzianum* was combined with the compost (T9) than *T. harzianum* as a stand-alone treatment (T8); the *T. harzianum* treatment (T8) was unable to significantly reduce the disease. The commercial products, FZB24 and FZB42, were not effective in suppressing the disease, either as a stand-alone treatment or in combination with the compost.

3.4.6 Analysis of anthocyanins

The influence of oak–bark compost, biological agents and the commercial products on the accumulation of anthocyanin was determined. The anthocyanin content was significantly higher in the plants that were treated with FZB24 (T4), FZB42 (T6), and *T. harzianum* (T8) compared to the untreated control (Fig. 5). In the combined treatment assays, only treatments containing *T. harzianum* (T9, T10 and T11) had significantly higher anthocyanin content over the untreated control (T0) (Fig. 5). Interestingly, the plants with higher accumulation of anthocyanin pigments appeared to have much darker leaves.

3.4.7 Detection of pathogen and biological agent DNA in rhizosphere soil and plant material

The presence of *P. infestans,* as well as the bio-agents and commercial products, was determined in the leaf tissue, root and rhizosphere soil for all treatments using specific DNA markers. *Phytophthora infestans* was detected in all *P. infestans*-inoculated plants (i.e., in both the whole plant and detached leaf assays). *P. infestans* was not detected in the untreated control plants for either assay. The biological agents, *B. subtilis* subsp. *subtilis* and *T. harzianum,* and the commercial products, FZB24 and FZB42, were detected in both roots and soil from the plants treated with the corresponding microbe. None of the microbes were detected in the extractions from leaves of the treated plants.

3.4.8 Screening of microbial community in oak–bark compost

To determine the presence of microbes in the oak–bark compost, the microbial community of the compost was screened. Multiple clones were sequenced. ITS amplicons corresponded to: *Antennariella placitae* Cheewangkoon and Crous, *Mortierella elongata* Linnem. and *Phialophora cyclaminis* J.F.H. Beyma. 16S rDNA amplicons corresponded to *Enterobacter cloacae* Hormaeche and Edwards, *Paenibacillus validus* Ash et al. and uncultured bacteria. This indicated that additional microbes may have played a role in disease suppression in treatments containing oak–bark compost.

3.5 Discussion

The present study showed that the oak–bark compost not only promotes plant growth, but also protects plants when exposed to *P. infestans*. The use of compost has become very popular as a cultural practice to improve soil health, promote growth and suppress disease. Composts are made from different source materials and, therefore, depending on the type of the compost, their effect on plant growth and/or disease suppression can vary (Termorshuizen et al. 2006). The influence of composts in suppression of soilborne pathogens, such as fungi and oomycetes is quite well-known (Hoitink and Boehm 1999; Termorshuizen et al. 2006; Bahramisharif et al. 2013; Tewoldemedhin et al. 2015). The use of soil amendments to control foliar pathogens is, however, not widely studied. A few studies have shown that non-aerated compost teas, which are fermented watery extracts, were able to inhibit the growth of *Ralstonia solanacearum* (Smith) Yabuuchi et al. in greenhouse trials and *Alternaria solani* Sorauer*, Botrytis cinerea* Pers. and *P. infestans* in *in vitro* assays (Kone et al. 2010; Mengesha et al. 2017). However, this study showed that compost as a stand-alone soil treatment has the potential to protect tomato plants from *P. infestans.*

Several factors can contribute to growth promotion and plant protection by compost. These factors can be divided into direct and indirect mechanisms. For example, soil augmented with compost may directly supply limiting nutrients for the plant. Likewise, compost may alter the soil microbe interactions and indirectly lead to protection against harmful pathogens. This can be through competition or antibiosis,

hyperparasitism and ISR (Hoitink and Boehm 1999). One of the most significant indirect modes of action is likely through modification of soil microbial activity and composition (Noble and Coventry 2005). In this study, we detected known endophytes in the oak–bark compost. The antimicrobial activity and potential of endophytes to control disease have been reported for several plant pathogens, including *P. infestans* in tomato (Kim et al. 2007; Miles et al. 2012). We recovered fungal species including *A. placitae*, *M. elongata* and *P. cyclaminis* in the oak–bark compost. *Antennariella placitae* has been shown to have significant antagonistic activity against *Ustilagonoidea virens* (Cooke) Takah on rice (Andargie et al. 2017). *Mortierella elongate* is usually found in soil, and has been reported to improve soil health and increase regulation of plant growth hormones (Li et al. 2018). *Phialophora cyclaminis* has been isolated from the rhizosphere of common oak and has been shown to have antifungal activity (Kaneto et al. 1993; Kwaśna 2001). We also detected bacterial species including *E. cloacae* and *P. validus* in the compost. *Enterobacter cloacae* is a PGPR and has been reported to be an effective biocontrol agent against soilborne pathogens such as *Pythium ultimum* Trow and *Phytophthora capsici* L. (Nelson and Maloney 1992; Toh et al. 2016). This bacterial agent has also been shown to enhance tomato resistance to *R. solanacearum* (Upreti and Thomas 2015). *Paenibacillus validus,* isolated from composts, has been reported to have cellulase and ligninase activities which are important for the composting process (Hemati et al. 2018). This bacterium has been shown to reduce the number of live *Candidatus* Liberibacter *asiaticus* (Citrus Huanglongbing pathogen) cells (Trivedi et al. 2011). In our study, oak–bark compost enhanced plant resistance to *P. infestans* in whole plant and detached leaf assays. This may be linked to the activation of ISR or the production of metabolites by endophytes present in the compost. Another factor that can contribute in the success of plant protection is the use of a large volume of composts (>15%) (Noble and Coventry 2005; Bahramisharif et al. 2013), due to an increase in microbial community. In our study, we used a 25% (v/v) of oak–bark compost.

Plant growth and protection were also present in treatments with *B. subtilis* subsp. *subtilis.* This Gram-positive bacterial strain is one of the major PGPRs that has shown promise for increasing crop yields. A number of mechanisms for plant growth promotion and pathogen protection have been proposed for *B. subtilis* subsp. *subtilis,* including the production of phytohormones, delivery of nutrients and stimulation of the

ISR (Beneduzi et al. 2012; Pieterse et al. 2014). In prior studies on tomato seedlings, *B. subtilis* has been shown to be effective in promoting growth which may be due to an increase in plant hormone production such as indole-3-acetic acid (IAA) and gibberellic acid (GA_3) (Chowdappa et al. 2013; Cendales et al. 2017). In our study, plants treated with *B. subtilis* subsp. *subtilis* were 2.5-fold larger in biomass. Furthermore, the growth promotion was significantly enhanced when *B. subtilis* subsp. *subtilis* was combined with the compost. A recent study by Rao et al. (2017) found that *B. subtilis* enriched vermicompost treatment increase carrot (*Daucus carota* subsp. *sativus* Hoffm.) yields by 28.8%.

Likewise, *B. subtilis* proved to be effective for enhancing plant resistance under biotic stress. In our study, tomato plants treated with *B. subtilis* were resistant to the pathogen not only in the whole plant assays, but also in detached leaf assays. This indicates that the beneficial effect was most likely through a systemic plant response, because *B. subtilis* was not detected in the detached leaf tissue, where the pathogen challenge took place. Plant resistance was further enhanced by the application of *B. subtilis* subsp. *subtilis* enriched oak–bark compost in both whole plant and detached leaf assays. This may be due to the improvement in root colonization by the bio-agent, as compost provides additional nutrition for the bacteria. The application of combined *B. subtilis* and vermicompost has been shown to be effective in the reduction of nematode population and soft rot disease incidence in carrot (Rao et al. 2017). The combination of composts and biological agents has been reported for a few soilborne pathogens and *P. capsici* on several crops including beans (*Phaseolus vulgaris* L.), onion (*Allium cepa* L.), pepper (*Capsicum annuum* L.), rooibos (*Aspalathus linearis* (N.L. Burm.) R. Dahlgr.) and turf grass (Nakasaki et al. 1998; Chae et al. 2006; Coventry et al. 2006; Pugliese et al. 2011; Bahramisharif et al. 2013). The co-inoculation of compost with *B. subtilis* subsp. *subtilis* could enhance the consistency in disease suppression, as also reviewed previously (Noble 2011). Here, anthocyanin accumulation in leaflet of plants treated with either *B. subtilis* subsp. *subtilis* or in combination with the compost was not enhanced. Anthocyanins are commonly upregulated in response to plant stress caused by biotic or abiotic factors (Dixon and Paiva 1995; de Vries et al. 2018). However, similar to this study, Yoon et al. (2015) found a reduction in the total anthocyanin content after black rice bran was fermented with *B. subtilis.* Likewise, the anthocyanin level was decreased in *B. subtilis* fermented

pigeon pea (Lee et al. 2015). This may be due to the hydrolysis of anthocyanin glycosides by β-glucosidase produced by *B. subtilis* (Asha et al. 2015).

Treatment with *Trichoderma harzianum* stimulated plant growth and protected plants from *P. infestans*, but only in whole plant assays. The plant protection by *T. harzianum* was not as great as when plants were treated with *B. subtilis* subsp. *subtilis*, in both whole plant and detached leaf assays. In contrast, *T. harzianum* has been reported to have greater potential for suppressing late blight disease incidence and severity in potato than *B. subtilis* (Wharton et al. 2012). The mode of action for growth promotion and plant protection by *T. harzianum* in whole plant assays may involve mycoparasitism, antibiotic production, competition, enzyme biosynthesis or ISR (Howell 2003). In this study, the level of anthocyanin was elevated in treatments that included *T. harzianum*. In other studies, plants exposed to volatiles of *Trichoderma* showed elevated levels of anthocyanin levels and were more resistant to *Botrytis cinerea* and *Alternaria brassicicola* (Schwein.) Wiltshire (Kottb et al. 2015). The application of *T. harzianum* in combination with oak–bark compost negatively affected the root growth and resulted in significantly higher disease severity in both whole plant and detached leaf assays. Likewise, on other crops, the co-inoculation of *T. harzianum* with compost was shown to be ineffective at suppressing *P. ultimum* in cucumber and *Phytophthora nicotianae* Breda de Haan in tomato (Pugliese et al. 2011). One hypothesis for the negative impact in the combined treatment is that *T. harzianum* competes with or acts antagonistically on the beneficial members of the microbial community in the compost, leading to an increase in disease severity. On the other hand, the co-inoculation of *T. harzianum* and compost has been reported to improve saline soil quality (Mbarki et al. 2017). In the current study, it was notable that the negative impact of the combined *T. harzianum* and compost treatment could be nullified if *B. subtilis* subsp. *subtilis* was also added, suggesting that this bacterial agent is insensitive to *T. harzianum*.

Variation in tomato growth and protection was observed for the two commercial products, FZB24 and FZB42. In general, FZB24 positively impacted growth more than FZB42. Previously, Gül et al. (2008) showed that the two strains of *B. amyloliquefaciens*, FZB24 and FZB42, increased tomato yield by nearly 10% in the

open hydroponic system; but, they both did not affect plant growth in the closed hydroponic system. Tryptophan-dependent synthesis of IAA has been implicated for the stimulation of plant growth by *B. amyloliquefaciens* (Idris et al. 2004, 2007). The FZB24 product was also better at plant protection than FZB42 in whole plant assays. However, none of these products were able to suppress late blight in detached leaf assays, which may be explained by the failure of these products to induce systemic defense in the host. The use of FZB24 has been reported to be effective in reducing disease caused by the oomycete *Pythium aphanidermatum* (Edson) Fitzp. at early stages in a hydroponic system of tomato (Grosch et al. 1999). In tomato plants, FZB24 product has also been shown to be effective against Fusarium crown and root rot (Myresiotis et al. 2012). Furthermore, FZB24 was tested against *Tilletia tritici* (Bjerk.) G. Winter, the causal agent of common bunt in wheat, and showed some beneficial activity under controlled conditions, but not in the field (Koch et al. 2006). The FZB42 product has been found to have antifungal activity towards *Fusarium graminearum* Schwabe which causes Fusarium head blight in cereal crops (Gu et al. 2017). The mechanisms involved in disease suppression by these products may be related to the enhancement of plant physical status through expression and upregulation of plant's defense compounds and genes, leading to plant stress-resistance, disease-resistance and growth (Xie et al. 2017). In the current study, FZB24 and FZB42 significantly increased anthocyanin content in tomato plants. The use of *Bacillus amyloliquefaciens* has been shown to significantly improve growth, yield and quality of strawberry fruits due to an increase in the level of natural antioxidants such as anthocyanins (Rahman et al. 2018). Other factors that can possibly be associated with disease suppression are the production of antifungal compounds such as Bacillomycin D (Gu et al. 2017) or the secretion of proteins such as acetolactate synthase (AlsS) by *B. amyloliquefaciens* that elicit plant innate immunity (Kierul et al. 2015). The co-inoculation of oak–bark compost and the commercial products resulted in an increase in plant growth but did not enhance plant protection further, compared to each product alone. It was also notable that compared to the untreated control, compost significantly decreased the level of anthocyanin when combined with the commercial products.

In conclusion, this study showed that oak–bark compost as a standalone treatment or in combination with *B. subtilis* subsp. *subtilis* and commercial products can be effective in enhancing tomato growth and resistance under biotic stress conditions. The

combination of the compost and *B. subtilis* subsp. *subtilis* showed the greatest promise for obtaining better growth and more effective and consistent plant protection, although this did not correspond to higher levels of anthocyanin in leaves. Future research should determine the mechanistic basis for plant growth promotion and protection in the combination treatment of oak–bark compost with *B. subtilis* subsp. *subtilis.*

3.6 Author contribution statement

AB and LER conceived and designed research. AB conducted experiments and analyzed data. AB wrote the manuscript. All authors read and approved the manuscript.

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3.9 Tables

Table 1. The 12 treatments that were used to test for growth promotion and disease suppression.

Treatments	Biological control				
	Compost amended	Biological agents		Commercial products	
		Bacillus subtilis subsp. *subtilis*	*Trichoderma harzianum*	FZB24	FZB42
T0/ Control	…	…	…	…	…
T1	×	…	…	…	…
T2	…	×	…	…	…
T3	×	×	…	…	…
T4	…	…	…	×	…
T5	×	…	…	×	…
T6	…	…	…	…	×
T7	×	…	…	…	×
T8	…	…	×	…	…
T9	×	…	×	…	…
T10	×	×	×	…	…
T11	×	×	×	×	×

Table 2. The universal and species-specific primers used in PCR analyses.

Primers	Sequence 5'–3'	References
ITS6-F	GAAGGTGAAGTCGTAACAAGG	Cooke et al. (2000)
ITS4-R	TCCTCCGCTTATTGATATGC	White et al. (1990)
*COX*2-F	GGCAAATGGGTTTTCAAGATCC	Hudspeth et al. (2000)
*COX*2-R	CCATGATTAATACCACAAATTTCACTAC	Hudspeth et al. (2000)
V5F primer (785F)	GGATTAGATACCCTGGTA	Arenz et al. (2015)
V6R primer (1064R)	CGACRRCCATGCANCACCT	Arenz et al. (2015)
CBS354-F	TGAAGAACGCAGCGAAATGC	Current study
CBS354-R	GCGAGTGTGCAAACTACTGC	Current study
DSM10-F	CCACACTGGGACTGAGACAC	Current study
DSM10-R	ACTTAAGAAACCGCCTGCGA	Current study
FZB-F	GTGAGGTAACGGCTCACCAA	Current study
FZB-R	GTGTCTCAGTCCCAGTGTGG	Current study

Table 3. Analyses of variance (ANOVA) for the effect of 12 treatments (T0–T11) on plant growth and necrotic area in whole plant and detached leaf assays.

Parameter	**df**[a]	**MS**[b]	**F**	**SL**[c]
Root length	11	0.025	6.029	< 0.0001
Shoot length	11	0.003	5.774	< 0.0001
Fresh weight	11	0.017	7.424	< 0.0001
Whole plant assay	11	0.173	4.161	< 0.0001
Detached leaf assay	11	0.079	5.282	< 0.0001
Anthocyanin content	11	0.045	3.190	0.002

[a] Degrees of freedom

[b] Mean squares

[c] Significant level of the *F* ratio

3.10 Figures

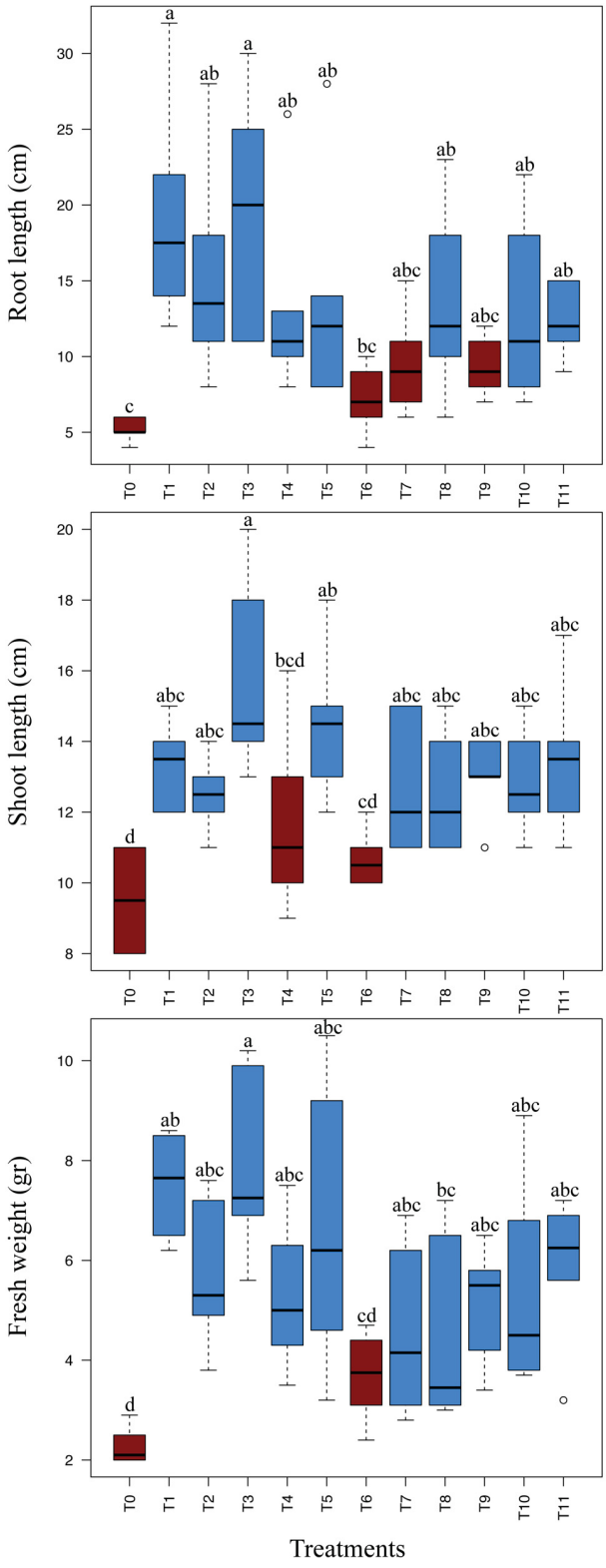

Fig. 1. Effect of the 12 treatments on root length, shoot length and fresh weight of tomato plants. The treatments were as follows: T0 (untreated control), T1 (compost),

T2 (*B. subtilis* subsp. *subtilis*), T3 (compost + *B. subtilis* subsp. *subtilis),* T4 (FZB24), T5 (compost + FZB24), T6 (FZB42), T7 (compost + FZB42), T8 (*T. harzianum*), T9 (compost + *T. harzianum*), T10 (compost + *B. subtilis* subsp. *subtilis* + *T. harzianum*), T11 (compost + *B. subtilis* subsp. *subtilis* + *T. harzianum* + FZB24 + FZB42). Data are from three replicates over two trials. The treatments that differ significantly from the untreated control are indicated in blue and the treatments that do not differ significantly from untreated control are indicated in red. Boxplots with the same letters do not differ significantly at $P = 0.05$.

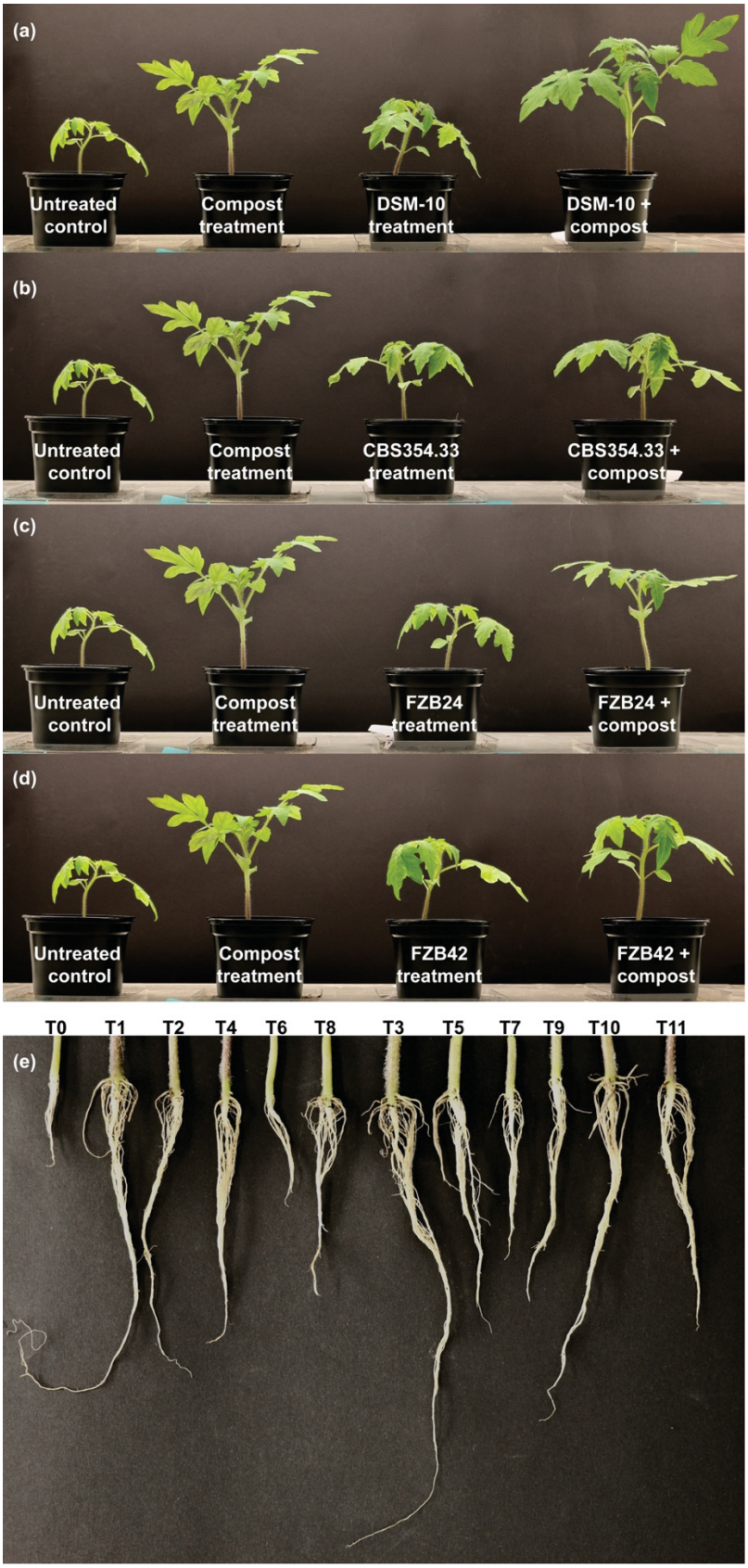

Fig. 2. Effect on tomato growth of treatments with **a)** *B. subtilis* subsp. *subtilis* (DSM-10), **b)** *T. harzianum* (CBS354.33), **c)** FZB24 and **d)** FZB42. **e)** Effect of the 12 treatments on root growth.

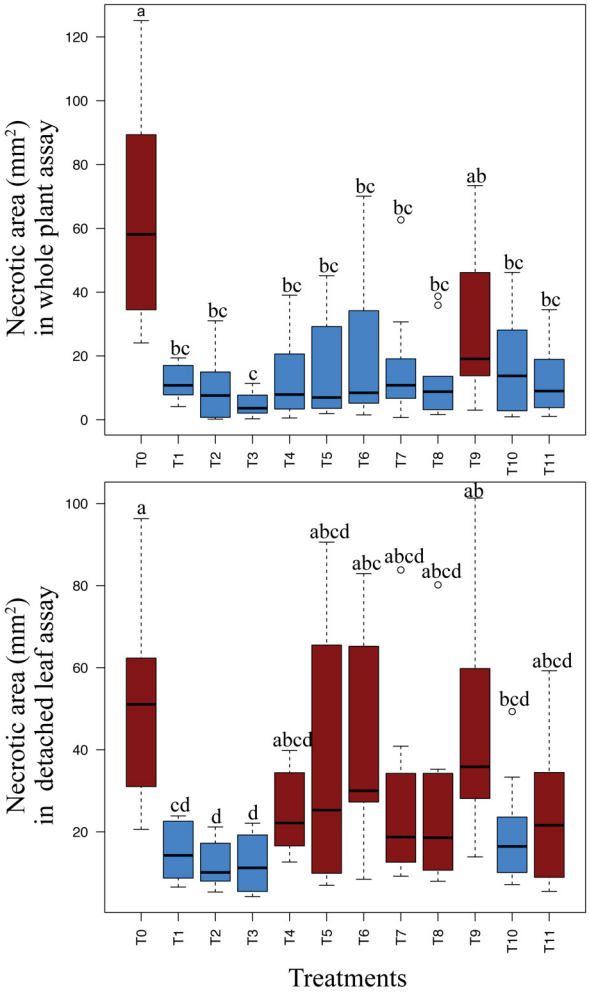

Fig. 3. Necrotic area for 12 treatments (T0–T11) in the whole plant and detached leaf assays. Data are from five replicates over two trials. The treatments that differ significantly from the untreated control are indicated in blue and the treatments that do not differ significantly from untreated control are indicated in red. Boxplots with the same letters do not differ significantly at $P = 0.05$.

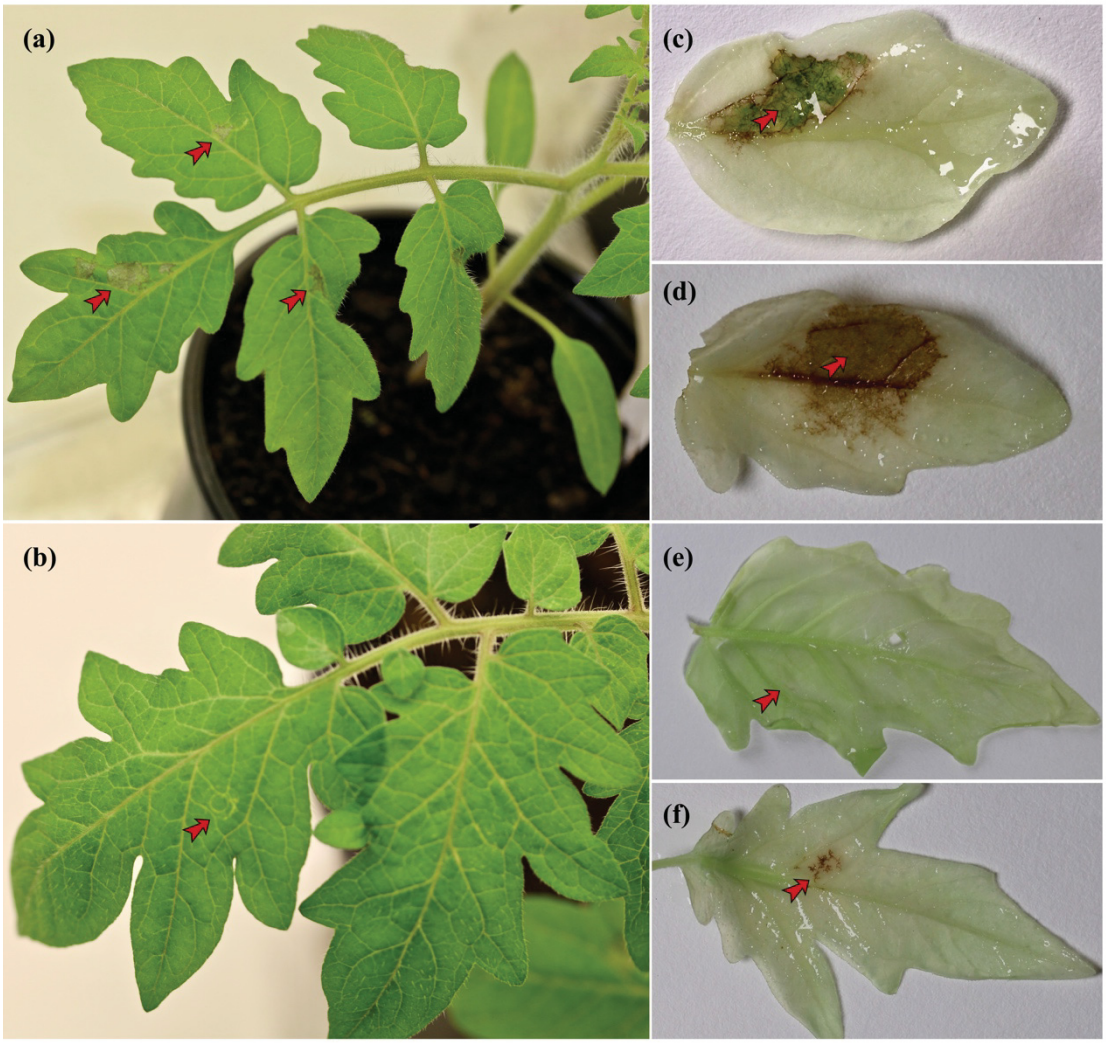

Fig. 4. Symptoms of 4-week-old Moneymaker tomato plants from infection by *P. infestans* isolate, D12-2. The red arrows indicate the location of infiltration / loading of *P. infestans* zoospores. **a)** The tomato seed planted in un-amended soil. **b)** The seed planted in oak–bark-compost-amended soil, combined with *B. subtilis* subsp. *subtilis.* **c** A bleached leaflet of an untreated control plant in the whole plant assay. **d)** A bleached leaflet of an untreated control plant in detached leaf assay. **e)** A bleached leaflet of the plant that was treated with a combination of oak–bark compost and *B. subtilis* subsp. *subtilis* in whole plant assay. **f)** A bleached leaflet of the plant that was treated with a combination of oak–bark compost and *B. subtilis* subsp. *subtilis* in the detached leaf assay.

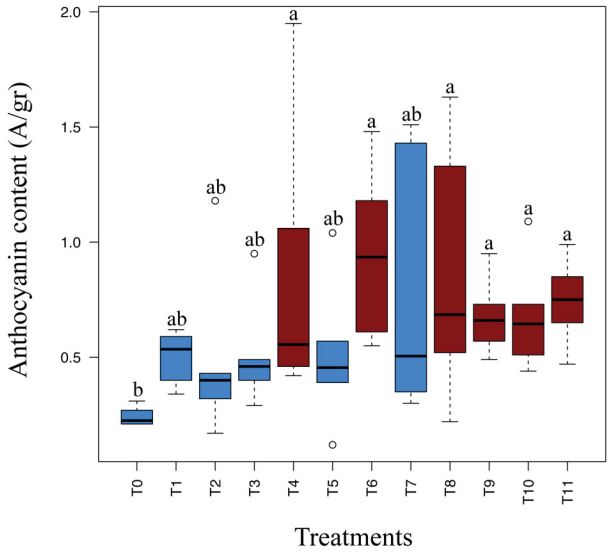

Fig. 5 Effect of the 12 treatments on anthocyanin accumulation in tomato leaflets. Six biological replicates were analyzed for each treatment. Anthocyanins (A) were evaluated by measuring the absorbance at 530 and 657 nm. The treatments that differ significantly from the untreated control are indicated in red and the treatments that do not differ significantly from untreated control are indicated in blue. Boxplots with the same letters do not differ significantly at $P = 0.05$

APPENDIX 1

JOURNAL VERSION OF CHAPTER 3

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ORIGINAL ARTICLE

Efficacy of biological agents and compost on growth and resistance of tomatoes to late blight

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Abstract

Main conclusion **This study identified biocontrol measures for improving plant quality and resistance under biotic stress caused by the most devastating pathogen in tomato production.**

The management of plant diseases are dependent on a variety of factors. Two important variables are the soil quality and its bacterial/fungal community. However, the interaction of these factors is not well understood and remains problematic in producing healthy crops. Here, the effect of oak–bark compost, *Bacillus subtilis* subsp. *subtilis*, *Trichoderma harzianum* and two commercial products (FZB24 and FZB42) were investigated on tomato growth, production of metabolites and resistance under biotic stress condition (infection with *Phytophthora infestans*). Oak–bark compost, *B. subtilis* subsp. *subtilis*, and *T. harzianum* significantly enhanced plant growth and immunity when exposed to *P. infestans*. However, the commercial products were not as effective in promoting growth, with FZB42 having the weakest protection. Furthermore, elevated levels of anthocyanins did not correlate with enhanced plant resistance. Overall, the most effective and consistent plant protection was obtained when *B. subtilis* subsp. *subtilis* was combined with oak–bark compost. In contrast, the combination of *T. harzianum* and oak–bark compost resulted in increased disease severity. The use of compost in combination with bio-agents should, therefore, be evaluated carefully for a reliable and consistent tomato protection.

Keywords Biocontrol · *Phytophthora infestans* · Plant–microbe interactions · Plant resistance · Secondary metabolites · Soil microbial community

Introduction

Cultivated tomato (*Solanum lycopersicum* L.) has a global production of 3.7 million hectares and is one of the most valuable agricultural crops worldwide (fao.org). However, tomatoes and nearly every crop species are also susceptible to a variety of pathogens that reduce both yield and quality. Late blight is the most devastating disease worldwide of both tomato and potato (Agrios 2005). Annual crop losses from late blight are estimated at over five billion USD (Judelson and Blanco 2005; Haverkort et al. 2009). Late blight is caused by *Phytophthora infestans* (Mont.) de Bary. This pathogen belongs to the Oomycota, a distinct lineage of filamentous eukaryotes which are fungus-like. This pathogen infects multiple plant species in the Solanaceae, including potato and tomato (Fry et al. 2015). In the mid-19th century, *P. infestans* devastated the potato crop and caused the Irish potato famine (Fry et al. 2015). To this day, disease management remains difficult and requires integrated management strategies.

Late blight affects the leaves, stems, and fruits of tomato and can cause total crop loss within as little as 2 weeks. The most effective control of late blight is by chemical fungicides. Phenylamide fungicides, such as mefenoxam, have been used against *P. infestans* and provide an effective disease suppression (Saville et al. 2015). However, chemical applications have detrimental outcomes to environmental and human health (Schummer et al. 2012a, b). Another

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major problem with the use of fungicides is the evolution of fungicide resistance in *P. infestans* populations (Taylor et al. 2002; Matson et al. 2015). Therefore, fungicides cannot be used continuously and do not represent a sustainable method of control. To maintain crop yields, without damaging the environment and human health, organic amendments and biocontrol agents have been explored as alternatives to chemical fungicides.

In horticulture, the application of compost to soil improves soil structure and plant root growth and results in an overall increase in yield of several crops, such as tomato (Gutierrez-Miceli et al. 2007). Furthermore, compost provides essential plant nutrients such as nitrogen, phosphorus, and calcium and thereby reduces the need for synthetic fertilizers (Lewis et al. 1992; Iqbal et al. 2010). Composts can be made of raw feedstocks such as yard trimmings, food waste, manure, tree leaves/bark and worm castings (Termorshuizen et al. 2006). The antagonistic and biological effect of compost for disease suppression is quite well-known. Compost has been used as an organic treatment for disease suppression against many soilborne pathogens, including oomycete species, *Rhizoctonia solani* Kühn and *Fusarium* species in tomato, cauliflower, rooibos, oats, lupin, pine, and flax (Termorshuizen et al. 2006; Bahramisharif et al. 2013; Tewoldemedhin et al. 2015; Lamprecht and Tewoldemedhin 2017). Composts, such as non-aerated compost teas, have also shown significant suppressive effects on foliar pathogens, where mycelial growth of *P. infestans* was completely inhibited in vitro (Kone et al. 2010). However, information is limited on the use of compost to control *P. infestans* in greenhouse or field conditions. Various factors may influence disease suppression by composts and thus, the effect of compost is not always consistent. These factors include compost composition, microbial biomass, the rate of application and maturity (Termorshuizen et al. 2006; Janvier et al. 2007). On the other hand, the application of some composts may be problematic, especially those rich in saline, which have been shown to enhance oomycete disease severity (Hoitink et al. 1997). Therefore, the use of composts and their nutrient content must be carefully evaluated to achieve consistent plant growth and disease suppression.

Biological agents (bio-agents), defined as living organisms, can significantly lower the density of plant pathogens (O'Brien 2017). Biological control has therefore become very popular as a non-chemical alternative to control late blight disease. In the last three decades, numerous bacterial bio-agents have been evaluated for their ability to suppress *P. infestans*. These include: *Bacillus amyloliquefaciens* Priest et al., *Bacillus cereus* Frankland and Frankland, *Bacillus pumilus* Meyer and Gottheil, *Bacillus subtilis* Ehrenberg, and *Pseudomonas fluorescens* Migula (Yan et al. 2002; An et al. 2010; Chowdappa et al. 2013). *Bacillus* species have shown great potential to promote plant growth and suppress late blight in tomato. Kabir et al. (2013) evaluated 125 different soil microbes and described six strains of *Bacillus* that suppressed late blight by more than 60% on culture plates and *in planta*. They also showed that these bio-agents are able to enhance plant growth. Some of these bio-agents are known as plant growth promoting rhizobacteria (PGPRs). Two of the most effective PGPRs are *B. subtilis* and *B. amyloliquefaciens*. They are both Gram-positive bacteria, commonly found in soil. The PGPRs colonize the root and promote plant growth and most importantly enhance protection against plant pathogens. Furthermore, *B. subtilis* and *B. amyloliquefaciens* have been shown to stimulate the plant immune system by activating plant induced systemic resistance (ISR) and promote growth in several crops, including tomato (Kloepper et al. 2004; Chowdappa et al. 2013). *Bacillus subtilis* and *B. amyloliquefaciens* are both available as commercial products, marketed as Serenade® (*B. subtilis,* strain QST 713), FZB24®WG (*B. amyloliquefaciens,* strain FZB24) and RhizoVital® 42 (*B. amyloliquefaciens,* strain FZB42).

There are numerous fungal antagonists that are also available as potential biocontrol agents. The fungal agents that are capable of suppressing *P. infestans* include *Fusarium oxysporum* Schlecht. emend. Snyder and Hansen, *Pythium oligandrum* Dreschler and *Trichoderma* species (Kim et al. 2007; Horner et al. 2012; Yao et al. 2016). *Trichoderma harzianum* Rafai and *P. oligandrum* have been reported to suppress the pathogen through competition, promoting plant growth and antibiosis or through mycoparasitism (Benhamou et al. 1999; Benitez et al. 2004). Similar to PGPRs, *Trichoderma* species stimulate plant immunity which may result in an activation of ISR in plants. Several studies have reported that *T. harzianum* upregulated induced defense response in different plants, including maize and tomato (Martinez-Medina et al. 2013; Saravanakumar et al. 2016).

While a few studies have shown that the use of organic amendments such as compost in combination with non-pathogenic species or biocontrol agents could significantly improve the disease suppression caused by highly virulent soilborne pathogens (Hoitink and Boehm 1999; Bahramisharif et al. 2013), the complex and inconsistent management of biological control has not translated into widespread use in field crops (Ryan et al. 2004; Xu et al. 2011). In particular, the potential of a combination compost and biological control treatment in tomato protection has not been fully exploited. The aim of this study was to identify reliable methods for improving plant resistance under biotic stress in tomato production. We evaluated (1) the effect of biological agents and commercial products on tomato growth, stress response and protection, (2) the potential of oak–bark compost as a standalone treatment or in combination with bio-agents in plant growth, stress and protection, and (3)

whether a combination of the compost with the biological agents or the commercial products increases consistency.

Materials and methods

Biological agents

In this study, two biocontrol agents were tested for the ability to control late blight disease in tomato: *Bacillus subtilis* subsp. *subtilis* and *Trichoderma harzianum*. The *Bacillus subtilis* subsp. *subtilis* isolate DSM-10 was sourced from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). The *T. harzianum* isolate CBS 354.33 was obtained from the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). Furthermore, two commercial products containing *B. amyloliquefaciens* ssp. *plantarum*: FZB24®WG and RhizoVital® 42/FZB42 were used. Both products were purchased from ABiTEP—Biotech for Agriculture and Ecology (Berlin, Germany).

Isolation and sporulation of *Trichoderma harzianum*

The *T. harzianum* isolate CBS 354.33 was grown on Trichoderma-selective media (Williams et al. 2003). The culture was incubated at 24 °C for 30 days in darkness. For sporulation of the isolate, the culture was plated out onto potato-dextrose agar (PDA) and incubated at 24 °C for 20 days. Ten ml ddH_2O was added to each plate and the plates were carefully sealed and incubated at room temperature for up to 2 h. The spores were then harvested as previously described (Perelló et al. 2009). The spore concentration was measured with a hemacytometer and a suspension with a concentration of 5×10^8 spores per ml was prepared and used immediately.

Isolation and sporulation of *Bacillus* spp.

Bacillus subtilis subsp. *subtilis* isolate DSM-10 was received as a freeze-dried culture. Following the supplier's instructions, the dried pellet was rehydrated with 0.5 ml of nutrient broth. After 30 min of incubation at room temperature, the content was gently mixed and about half of the content was transferred into a 5 ml tube containing nutrient broth. The other half was streaked onto nutrient agar plates and used for storage. The broth cultures were incubated on rotary shaker at 200 rpm at 30 °C (Nakamura et al. 1999) until the logarithmic phase was reached. The OD_{600} value was calculated using a DeNovix DS-11 FX spectrophotometer (DeNovix Inc., Wilmington, DE, USA). The suspension was diluted to reach the concentration of 5×10^8 colony forming units per ml (CFU/ml).

The two commercial products, FZB24®WG and RhizoVital® 42/FZB42, contain living spores of *B. amyloliquefaciens* ssp. *plantarum*. These products were first diluted in ddH2O and 100 µl of the suspension was streaked on Luria–Bertani broth (LB) medium containing 1.5% agar. The cultures were incubated at 30 °C for 2 days and the bacterial cells were then harvested and grown in LB. The broth cultures were incubated at 30 °C overnight on rotary shaker (at 200 rpm). The spore concentration of 5×10^8 CFU/ml was prepared exactly as for DSM-10 and used immediately.

Biomass and plant height

To evaluate the effect of the treatments on plant growth, 3-week-old tomato plants were first carefully removed from the pots. The soil adhering to the roots was removed by gentle shaking and the remaining rhizosphere soil was brushed off into a 50 ml falcon tube for DNA extraction. The root was washed with water thoroughly to remove soil particles adhering to the root. Root and shoot length, as well as, fresh weight was then determined for all plants.

Efficacy of biocontrol

Twelve different treatments were evaluated for their ability to enhance plant growth and/or to suppress disease (Table 1). Each treatment had nine biological replicates, of which three were used to evaluate growth and six were used to evaluate disease suppression. The experiment was replicated two times. The replicates were carried out in a growth chamber under standard growing conditions for tomatoes. The

Table 1 The 12 treatments that were used to test for growth promotion and disease suppression

Treatments	Biological control				
	Compost amended	Biological agents		Commercial products	
		Bacillus subtilis subsp. *subtilis*	*Trichoderma harzianum*	FZB24	FZB42
T0/control	…	…	…	…	…
T1	×	…	…	…	…
T2	…	×	…	…	…
T3	×	×	…	…	…
T4	…	…	…	×	…
T5	×	…	…	×	…
T6	…	…	…	…	×
T7	×	…	…	…	×
T8	…	…	×	…	…
T9	×	…	×	…	…
T10	×	×	×	…	…
T11	×	×	×	×	×

replicates followed a randomized block design with the placement of the pots being changed every 7 days.

Five treatments contained un-amended soil (*Stender*® C-400 with Cocopeat) and seven treatments contained the same soil amended with 25% v/v of oak–bark compost. For all treatments, 11-cm diameter plastic pots were filled with approximately 1 kg of either soil or the soil amended with compost. One hole with a depth of 1.5 cm was made in the soil using 1 cm diameter sterile doweling rods. Directly before planting the tomato seed, 1 ml of biocontrol agent suspension was pipetted into the hole according to the treatment. Combined treatments received 1 ml suspension for all agents. Directly afterwards, one tomato seed (cv. Moneymaker) was planted into the hole and covered.

Isolation and sporulation of *P. infestans*

The highly virulent *P. infestans* isolate, D 12-2, was obtained from Francine Govers' Laboratory of Phytopathology (Wageningen University, The Netherlands). This isolate was grown on Rye B Agar (Caten and Jinks 1968). The medium was prepared with 60 g of rye grain soaked in ddH_2O for 24 h. The supernatant was then removed and 1 l ddH_2O was added. The mixture was then boiled for 2 h in a 2 l sterile beaker. The supernatant was filtered through cheesecloth and combined with the original supernatant. Then, 15 g of Bacto agar, 20 g of sucrose and 0.05 g beta-sitosterol were added to the supernatant and autoclaved at 15 psi for 20 min. Following incubation on plates at 18 °C for 20 days in darkness, 10 ml of cold ddH_2O was used to harvest *P. infestans* sporangia. A zoospore suspension was then prepared by placing the suspension at 4 °C for 2–4 h until the zoospores were released (de Vries et al. 2017). A suspension with a total concentration of 5×10^5 zoospores/ml was prepared for infection using a hemacytometer.

Plant biotic stress assays (whole plant and detached leaf infection assays)

Whole plant infections were done on 3-week-old tomato plants using artificial inoculation technique as follows: Ten µl of the *P. infestans* zoospore suspension (5×10^5 zoospores/ml) was carefully infiltrated into the extracellular space of five young leaves using a 1 ml needleless syringe. For the control plants, 10 µl of ddH_2O was infiltrated into five leaves using the same technique. The inoculated leaflets were collected after 5 days. Re-isolation was made from the leaflet and root fulfilling Koch's postulates.

In the detached leaf infection assay, five young leaves from 3-week-old plants were excised and placed in a Petri dish, containing a wet sterile paper towel. Ten µl of the zoospore suspension (5×10^5 zoospores/ml) was loaded onto the abaxial surface of detached leaves. For the control plants, 10 µl of ddH_2O was loaded. The Petri dishes were kept at 18 °C in the dark for 5 days.

Screening of necrotic lesions by *P. infestans*

For both the whole plant and detached leaf assays, all inoculated leaves were bleached using 100% EtOH for 72 h. After bleaching out the chlorophyll, the necrotic lesions were examined under a SteREO Discovery V8 binocular (AxioCam ICc 5 camera; Zeiss, Jena, Germany) and quantified with the ZEN lite 2012 software (Zeiss).

Defense-related compounds

Anthocyanins, phenolic flavonoid pigments, are synthesized by the phenylpropanoid pathway and may be induced in response to plant stress. To determine whether the compost or the bio-agents affected the level of anthocyanin production in the leaves, the anthocyanin content was evaluated as previously described (Lindoo and Caldwell 1978). Six biological replicates were used for each treatment.

Design of specific primers for *T. harzianum* and *B. subtilis* subsp. *subtilis*

The ITS sequence of *T. harzianum* (CBS 354.33; AF278790) and 16S rRNA sequences of *B. subtilis* subsp. *subtilis* (DSM-10; LN681568), *B. amyloliquefaciens* strains FZB24 (AY055219) and FZB42 (AY055221) were downloaded from GenBank (https://www.ncbi.nlm.nih.gov) and uploaded into Geneious® 11.0.2 (Kearse et al. 2012). Species-specific primers were designed for all sequences using the Geneious plugin Primer 3 2.3.7 (Rozen and Skaletsky 2000) (Table 2).

DNA extraction and PCR amplification of *P. infestans* and *T. harzianum* isolates

For positive controls of the PCR amplification, *Phytophthora infestans* was grown on 20% unclarified V8 Agar (200 ml V8 juice, 800 ml ddH_2O, 15 g agar, 2 g $CaCO_3$ and 0.05 g beta-sitosterol) for 14 days and *T. harzianum* was grown on PDA for 7 days. Mycelium from both were then harvested and genomic DNA (gDNA) was extracted using the extraction method described by Edwards et al. (1991).

For DNA amplification of *P. infestans*, the cytochrome *c* oxidase subunit II (*COX2*) region was used (Hudspeth et al. 2000; Table 2). For *T. harzianum,* DNA was amplified for the specific primers described above. Polymerase chain reaction (PCR) was conducted in a total volume of 20 µl consisting of Green GoTaq® Flexi Buffer, 2 U GoTaq® Flexi DNA Polymerase (Promega, Fitchburg, WI, USA), 1.25 mM $MgCl_2$, 0.1 mM dNTPs and 0.2 mM of each primer. The T100™ Thermal

Table 2 The universal and species-specific primers used in PCR analyses

Primers	Sequence 5′–3′	References
ITS6-F	GAAGGTGAAGTCGTAACAAGG	Cooke et al. (2000)
ITS4-R	TCCTCCGCTTATTGATATGC	White et al. (1990)
Cox2-F	GGCAAATGGGTTTTCAAGATCC	Hudspeth et al. (2000)
Cox2-R	CCATGATTAATACCACAAATTTCACTAC	Hudspeth et al. (2000)
V5F primer (785F)	GGATTAGATACCCTGGTA	Arenz et al. (2015)
V6R primer (1064R)	CGACRRCCATGCANCACCT	Arenz et al. (2015)
CBS354-F	TGAAGAACGCAGCGAAATGC	Current study
CBS354-R	GCGAGTGTGCAAACTACTGC	Current study
DSM10-F	CCACACTGGGACTGAGACAC	Current study
DSM10-R	ACTTAAGAAACCGCCTGCGA	Current study
FZB-F	GTGAGGTAACGGCTCACCAA	Current study
FZB-R	GTGTCTCAGTCCCAGTGTGG	Current study

Cycler (Bio-Rad, Hercules, CA, USA) was used for amplification of *P. infestans* with PCR condition as follows: initial denaturation at 95 °C for 3 min, 34 cycles of 95 °C for 30 s, annealing for 30 s at 60 °C, extension at 72 °C for 1.30 min, and final extension at 72 °C for 5 min. The PCR for *T. harzianum* was carried out with initial denaturation at 94 °C for 5 min, 32 cycles of 94 °C for 30 s, annealing for 30 s at 55 °C, extension at 72 °C for 1.30 min, and final extension at 72 °C for 7 min. Gel electrophoresis was analyzed by resolving PCR products in 1% agarose gel and DNA was stained using Midori Green Advance (Nippon Genetics Europe, Dueren, Germany).

DNA extraction and PCR amplification from bacterial isolates

The bacterial cells of DSM-10 were harvested from nutrient agar and then added into nutrient broth. For FZB24 and FZB42, the cells were harvested from LB medium. The broth media were incubated at 30 °C for overnight on rotary shaker at 200 rpm. Chromosomal DNA was then extracted using DNeasy® PowerLyzer® Microbial Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. For the amplification of 16S rDNA, the universal primers V5F and V6R (Arenz et al. 2015) and newly designed primers were used (Table 2). PCR was carried out in the T100™ Thermal Cycler (Bio-Rad) with initial denaturation at 94 °C for 9 min, following 30 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 45 s, and the final extension for 7 min at 72 °C. To conduct gel electrophoresis, PCR products were resolved in 1% agarose gel and DNA was visualized by staining with Midori Green Advance (Nippon Genetics Europe).

DNA extraction from rhizosphere soil and plant material

To evaluate relative abundance of different species in rhizosphere soil and plant material, gDNA was extracted from rhizosphere soil using DNeasy® PowerSoil® Kit (Qiagen), and roots and leaves using DNeasy® PowerPlant® Pro Kit (Qiagen) according to manufacturer's instructions. PCR was conducted for screening for the presence of *P. infestans* and the bio-agents using the same PCR conditions as described above.

Cloning and sequencing

To investigate the microbial community in oak–bark compost, gDNA was extracted from the compost using DNeasy® PowerSoil® Kit (Qiagen). PCR was conducted for amplification of DNA for the ITS and 16S rDNA regions with the same conditions as described above. The PCR product of compost's DNA was purified using peqGOLD Cycle-Pure kit (VWR, Peqlab, Radnor, PA, USA). The purified PCR product was then cloned using TOPO® TA cloning® kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. To screen the microbial community, sequencing analyses of the ITS and 16S rDNA regions of three clones were carried out by Eurofins Genomics DNA sequencing facility (Eurofins Genomics, Ebersberg, Germany). For the ITS region, the universal primers ITS6 (Cooke et al. 2000) and ITS4 (White et al. 1990) were used, and 16S rDNA region was amplified using primers V5F and V6R (Table 2).

Statistical analyses

To test for statistical differences between treatments, one-way ANOVA was performed for all the phenotypic measurements, including shoot and root length, plant fresh weight and necrotic area. Levene's test was conducted for homogeneity of two repeat trials (Levene 1961). Independent-samples *t* test was performed to compare the variances of all pairwise combinations. The Shapiro–Wilk's test was conducted to test for deviations from normality (Shapiro and

Wilk 1965). Tukey's HSD (Honest significance different) test was calculated for each of these phenotypic measurements as well as anthocyanin content to determine significant differences at 5% level (Tukey 1949). All the procedures were performed by IBM SPSS Statistics software (version 25).

Results

Growth promotion by oak–bark compost and bio-agents

The null hypothesis of the Levene's test could not be rejected ($P > 0.05$); therefore, the data from the two repeat trials were combined. The Shapiro–Wilk's test showed that growth data do not fit the normal distribution. Thus, before the analysis of variance, the data were transformed to meet the assumption of normality using logarithmic transformation. According to the one-way analysis of variance plant growth differed significantly across treatments (Table 3).

Treatments with oak–bark compost and certain bio-agents significantly increased tomato growth (Figs. 1 and 2). The compost-treated plants (T1) were the largest. Compared to the control plants (T0), plants grown with compost (T1) had 3.7-fold longer roots, 1.4-fold longer shoots and 3.3-fold greater fresh weight (Figs. 1, 2a–e). The two bio-agents, *B. subtilis* subsp. *subtilis* (T2) and *T. harzianum* (T8), significantly enhanced plant growth compared to the control (T0) (Figs. 1, 2a, b, e). The biomass was, however, larger by *B. subtilis* subsp. *subtilis* (T2) than *T. harzianum* (T3). The FZB24 product (T4) was better in promoting plant growth than FZB42 (T6). Treatment with FZB24 (T4) significantly improved root growth and fresh weight, while treatment with FZB42 (T6) did not significantly promote plant growth compared to the untreated control (T0) (Figs. 1, 2c–e).

Table 3 Analyses of variance (ANOVA) for the effect of 12 treatments (T0–T11) on plant growth and necrotic area in whole plant and detached leaf assays

Parameter	*df*[a]	MS[b]	*F*	SL[c]
Root length	11	0.025	6.029	< 0.0001
Shoot length	11	0.003	5.774	< 0.0001
Fresh weight	11	0.017	7.424	< 0.0001
Whole plant assay	11	0.173	4.161	< 0.0001
Detached leaf assay	11	0.079	5.282	< 0.0001
Anthocyanin content	11	0.045	3.190	0.002

[a]Degrees of freedom

[b]Mean squares

[c]Significant level of the *F* ratio

Growth promotion by combination treatments

The assays showed that the combination of the compost and bio-agents or commercial products can further increase plant growth (Figs. 1, 2). *B. subtilis* subsp. *subtilis* in combination with compost (T3) enhanced plant growth, compared to the stand-alone treatment with *B. subtilis* subsp. *subtilis* (T2) (Figs. 1, 2a). Compared to the control plants (T0), plants grown with a combination of oak–bark compost and *B. subtilis* subsp. *subtilis* (T3) had 3.8-fold longer roots, 1.6-fold longer shoots and 3.5-fold greater fresh weight (Figs. 1, 2a, e). Plants treated with FZB24 and compost (T5) had significantly longer shoots compared to the untreated control (T0) and had 1.2-fold longer shoots than in the standalone treatment with FZB24 (T4) (Figs. 1, 2c). Although FZB42 (T6) did not significantly stimulate plant growth compared to the untreated control, shoot length and fresh weight were significantly enhanced, when this commercial product was combined with the compost (T7) (Figs. 1, 2d).

Plant protection by oak–bark compost and bio-agents in whole plant assay

Based on Levene's test, variance from the two repeat trials were comparable and, thus data were combined. The normality test by Shapiro–Wilk was rejected for the necrotic area data and therefore, the data was transformed to meet the assumption of normality using logarithmic transformation. Strong evidence for significant interactions was observed across treatments (Table 1).

All of the treatments evaluated in this study (T1–T11), except treatment T9, protected tomato plants from the disease (Fig. 3), while the untreated control plants remained highly susceptible to *P. infestans* (Fig. 4a, c). Oak–bark compost, as a stand-alone treatment (T1), suppressed the disease by 82% on average. The highest suppression of late blight (85%) was achieved in the treatment with *B. subtilis* subsp. *subtilis* (T2), but *T. harzianum* (T8) and FZB24 (T4) also showed high disease suppression (80% and 79%, respectively). In contrast, FZB42 (T6) was the least effective of all treatments, averaging 70% disease suppression.

Plant protection by co-inoculation of oak–bark compost and bio-agents in whole plant assay

The biological agents, in combination with the compost, improved plant protection, but not for all of the treatments (Fig. 3). Late blight disease severity decreased, on average, by 8% when the compost was combined with *B. subtilis* subsp. *subtilis* (T3) (Fig. 4b, e). Furthermore, the variance was significantly lower in combination treatments of compost with *B. subtilis* subsp. *subtilis* (T3), indicating consistent disease control. However, the combination of *T.*

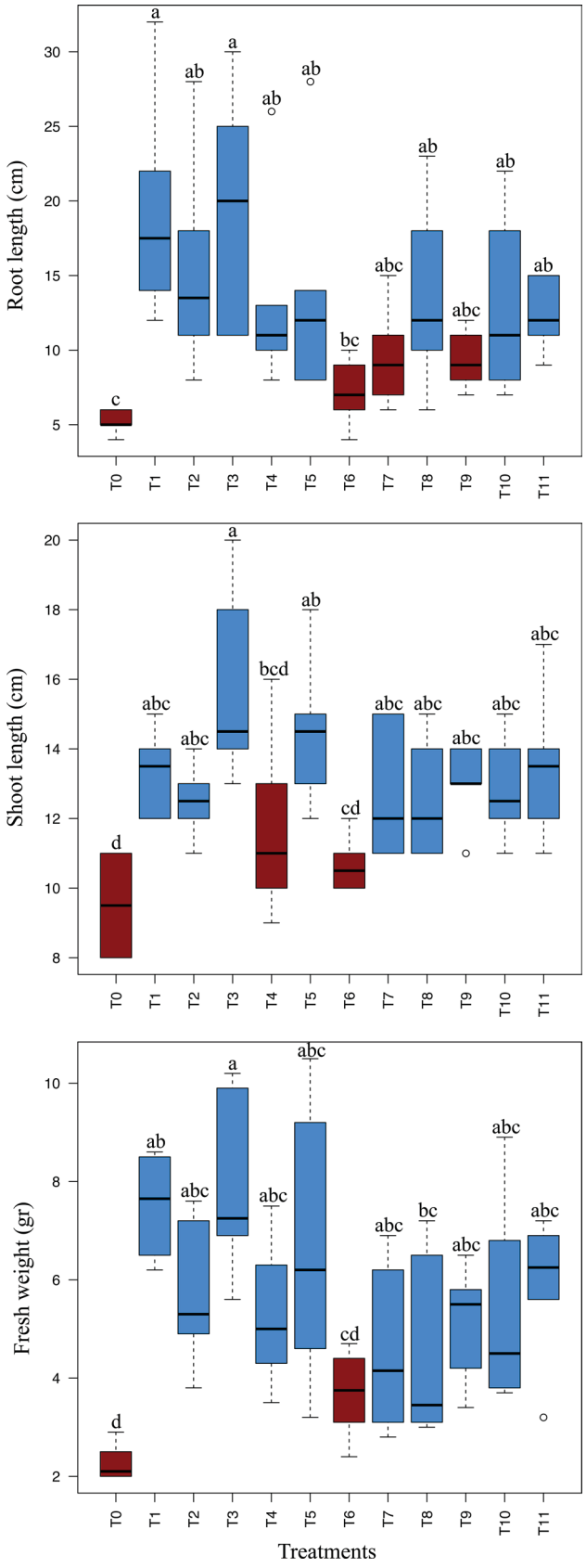

Fig. 1 Effect of the 12 treatments on root length, shoot length and fresh weight of tomato plants. The treatments were as follows: T0 (untreated control), T1 (compost), T2 (*B. subtilis* subsp. *subtilis*), T3 (compost + *B. subtlis* subsp. *subtilis),* T4 (FZB24), T5 (compost + FZB24), T6 (FZB42), T7 (compost + FZB42), T8 (*T. harzianum*), T9 (compost + *T. harzianum*), T10 (compost + *B. subtlis* subsp. *subtilis* + *T. harzianum*), T11 (compost + *B. subtlis* subsp. *subtilis* + *T. harzianum* + FZB24 + FZB42). Data are from three replicates over two trials. The treatments that differ significantly from the untreated control are indicated in blue and the treatments that do not differ significantly from untreated control are indicated in red. Boxplots with the same letters do not differ significantly at $P = 0.05$

harzianum with compost (T9) resulted in a 23% increase in disease severity compared to *T. harzianum* alone (T8). The addition of *B. subtilis* subsp. *subtilis* to the treatment including *T. harzianum* and compost (T10) increased the suppression by 16% relative to T9. Furthermore, the negative impact of the combination of compost and *T. harzianum* was nullified when the bio-agents and commercial products were combined (T11), a treatment which resulted in an average total suppression of 80%, compared to the untreated control (T0).

Plant protection in detached leaf assay

In the detached leaf assay, oak–bark compost (T1) suppressed late blight disease by 71% and by *B. subtilis* subsp. *subtilis* (T2) suppressed late blight disease by 76% compared to the untreated control plants (T0) (Fig. 3). Furthermore, in the combined treatments, only treatments T3 and T10 reduced late blight incidence compared to the untreated control (T0) (Figs. 3, 4f). Similar to the whole plant assays, the disease severity was higher when *T. harzianum* was combined with the compost (T9) than *T. harzianum* as a stand-alone treatment (T8); the *T. harzianum* treatment (T8) was unable to significantly reduce the disease. The commercial products, FZB24 and FZB42, were not effective in suppressing the disease, either as a stand-alone treatment or in combination with the compost.

Analysis of anthocyanins

The influence of oak–bark compost, biological agents and the commercial products on the accumulation of anthocyanin was determined. The anthocyanin content was significantly higher in the plants that were treated with FZB24 (T4), FZB42 (T6), and *T. harzianum* (T8) compared to the untreated control (Fig. 5). In the combined treatment assays, only treatments containing *T. harzianum* (T9, T10 and T11) had significantly higher anthocyanin content over the untreated control (T0) (Fig. 5). Interestingly, the plants with higher accumulation of anthocyanin pigments appeared to have much darker leaves.

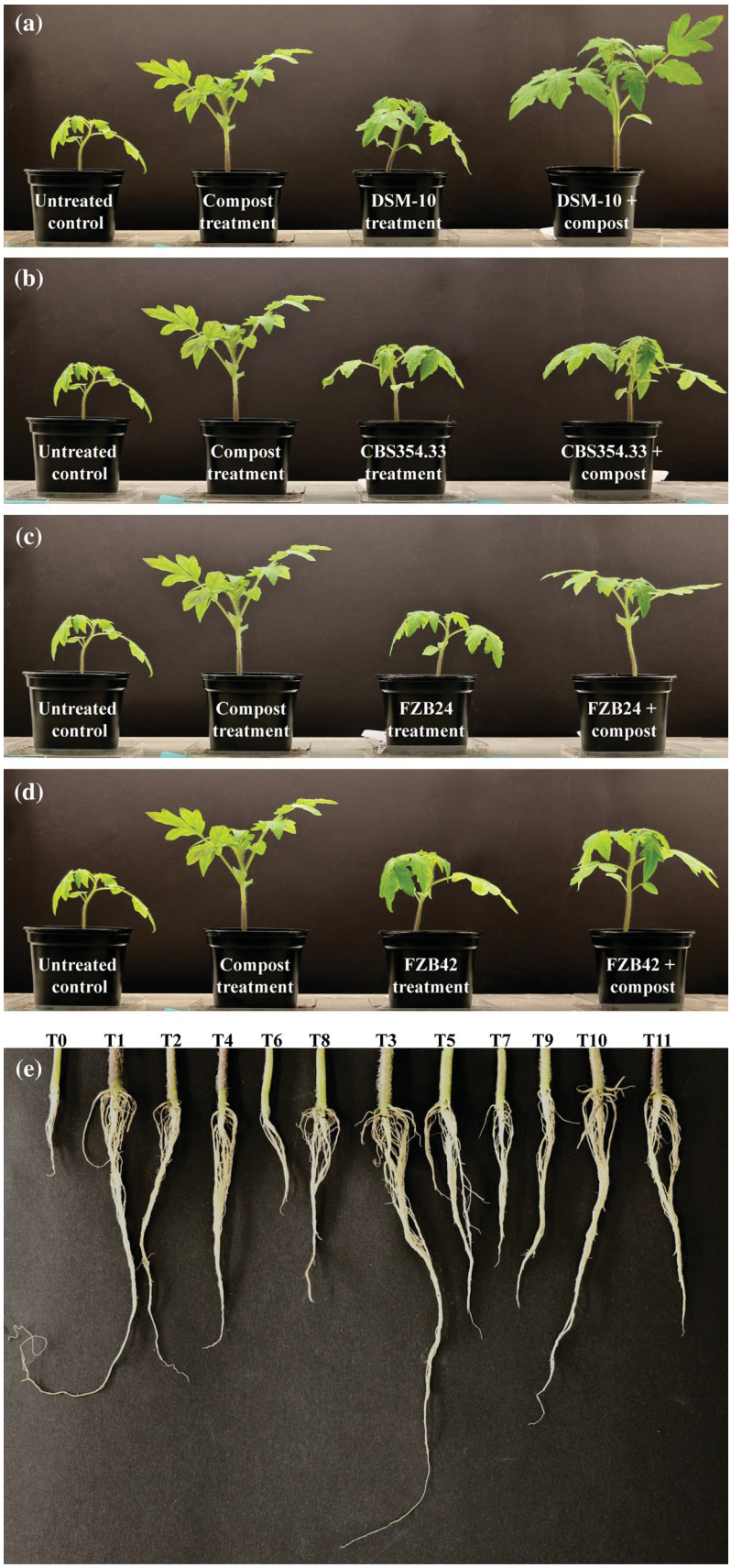

Fig. 2 Effect on tomato growth of treatments with *B. subtlis* subsp. *subtilis* (DSM-10, **a**); *T. harzianum* (CBS354.33, **b**); FZB24 (**c**) and FZB42 (**d**). **e** Effect of the 12 treatments on root growth

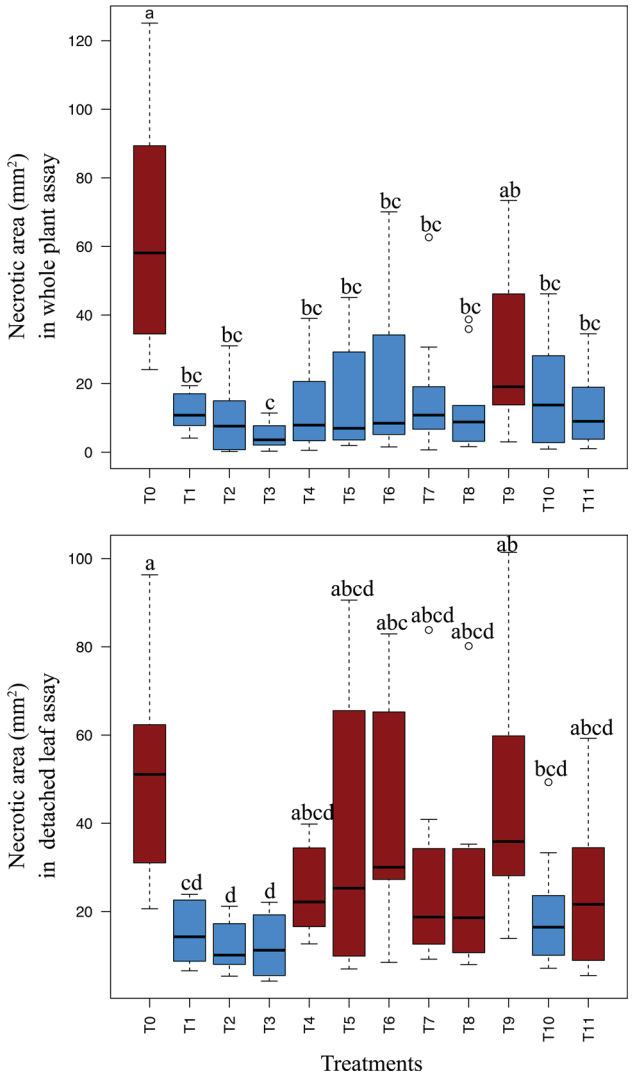

Fig. 3 Necrotic area for 12 treatments (T0–T11) in the whole plant and detached leaf assays. Data are from five replicates over two trials. The treatments that differ significantly from the untreated control are indicated in blue and the treatments that do not differ significantly from untreated control are indicated in red. Boxplots with the same letters do not differ significantly at $P = 0.05$

Detection of pathogen and biological agent DNA in rhizosphere soil and plant material

The presence of *P. infestans,* as well as the bio-agents and commercial products, was determined in the leaf tissue, root and rhizosphere soil for all treatments using specific DNA markers. *Phytophthora infestans* was detected in all *P. infestans*-inoculated plants (i.e., in both the whole plant and detached leaf assays). *P. infestans* was not detected in the untreated control plants for either assay. The biological agents, *B. subtilis* subsp. *subtilis* and *T. harzianum,* and the commercial products, FZB24 and FZB42, were detected in both roots and soil from the plants treated with the corresponding microbe. None of the microbes were detected in the extractions from leaves of the treated plants.

Screening of microbial community in oak–bark compost

To determine the presence of microbes in the oak–bark compost, the microbial community of the compost was screened. Multiple clones were sequenced. ITS amplicons corresponded to: *Antennariella placitae* Cheewangkoon and Crous, *Mortierella elongata* Linnem. and *Phialophora cyclaminis* J.F.H. Beyma. 16S rDNA amplicons corresponded to *Enterobacter cloacae* Hormaeche and Edwards, *Paenibacillus validus* Ash et al. and uncultured bacteria. This indicated that additional microbes may have played a role in disease suppression in treatments containing oak–bark compost.

Discussion

The present study showed that the oak–bark compost not only promotes plant growth, but also protects plants when exposed to *P. infestans*. The use of compost has become very popular as a cultural practice to improve soil health, promote growth and suppress disease. Composts are made from different source materials and, therefore, depending on the type of the compost, their effect on plant growth and/or disease suppression can vary (Termorshuizen et al. 2006). The influence of composts in suppression of soilborne pathogens, such as fungi and oomycetes is quite well-known (Hoitink and Boehm 1999; Termorshuizen et al. 2006; Bahramisharif et al. 2013; Tewoldemedhin et al. 2015). The use of soil amendments to control foliar pathogens is, however, not widely studied. A few studies have shown that non-aerated compost teas, which are fermented watery extracts, were able to inhibit the growth of *Ralstonia solanacearum* (Smith) Yabuuchi et al. in greenhouse trials and *Alternaria solani* Sorauer*, Botrytis cinerea* Pers. and *P. infestans* in in vitro assays (Kone et al. 2010; Mengesha et al. 2017). However, this study showed that compost as a stand-alone soil treatment has the potential to protect tomato plants from *P. infestans.*

Several factors can contribute to growth promotion and plant protection by compost. These factors can be divided into direct and indirect mechanisms. For example, soil augmented with compost may directly supply limiting nutrients for the plant. Likewise, compost may alter the soil microbe interactions and indirectly lead to protection against harmful pathogens. This can be through competition or antibiosis, hyperparasitism and ISR (Hoitink and Boehm 1999). One of the most significant indirect modes of action is likely through modification of soil microbial activity and

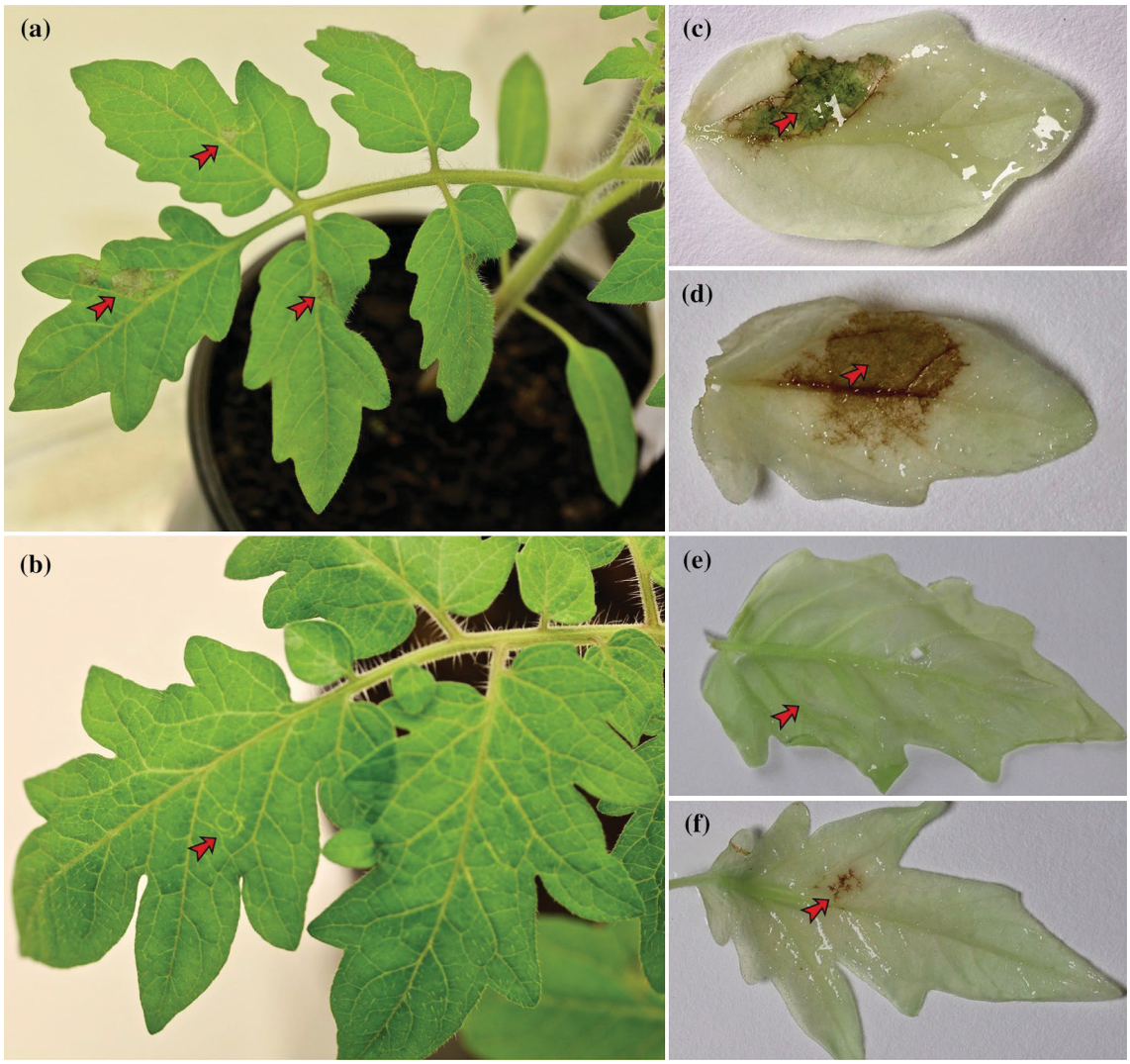

Fig. 4 Symptoms of 4-week-old Moneymaker tomato plants from infection by *P. infestans* isolate, D12-2. The red arrows indicate the location of infiltration/loading of *P. infestans* zoospores. **a** The tomato seed planted in un-amended soil. **b** The seed planted in oak–bark-compost-amended soil, combined with *B. subtilis* subsp. *subtilis*. **c** A bleached leaflet of an untreated control plant in the whole plant assay. **d** A bleached leaflet of an untreated control plant in detached leaf assay. **e** A bleached leaflet of the plant that was treated with a combination of oak–bark compost and *B. subtilis* subsp. *subtilis* in whole plant assay. **f** A bleached leaflet of the plant that was treated with a combination of oak–bark compost and *B. subtilis* subsp. *subtilis* in the detached leaf assay

composition (Noble and Coventry 2005). In this study, we detected known endophytes in the oak–bark compost. The antimicrobial activity and potential of endophytes to control disease have been reported for several plant pathogens, including *P. infestans* in tomato (Kim et al. 2007; Miles et al. 2012). We recovered fungal species including *A. placitae*, *M. elongata* and *P. cyclaminis* in the oak–bark compost. *Antennariella placitae* has been shown to have significant antagonistic activity against *Ustilagonoidea virens* (Cooke) Takah on rice (Andargie et al. 2017). *Mortierella elongate* is usually found in soil, and has been reported to improve soil health and increase regulation of plant growth hormones (Li et al. 2018). *Phialophora cyclaminis* has been isolated from the rhizosphere of common oak and has been shown to have antifungal activity (Kaneto et al. 1993; Kwaśna 2001). We also detected bacterial species including *E. cloacae* and *P. validus* in the compost. *Enterobacter cloacae* is a PGPR and has been reported to be an effective biocontrol agent against soilborne pathogens such as *Pythium ultimum* Trow and *Phytophthora capsici* L. (Nelson and Maloney 1992; Toh et al. 2016). This bacterial agent has also been shown to enhance tomato resistance to *R. solanacearum* (Upreti and Thomas 2015). *Paenibacillus validus,* isolated from composts, has been reported to have cellulase and ligninase activities which are important for the composting process (Hemati et al. 2018). This bacterium has been shown to

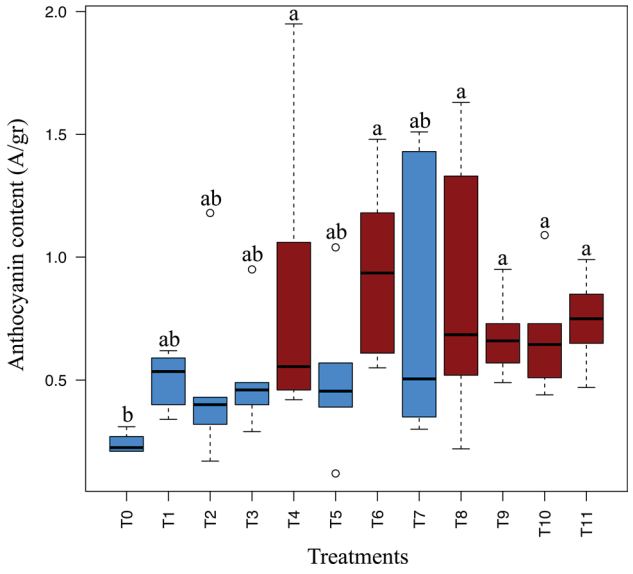

Fig. 5 Effect of the 12 treatments on anthocyanin accumulation in tomato leaflets. Six biological replicates were analyzed for each treatment. Anthocyanins (A) were evaluated by measuring the absorbance at 530 and 657 nm. The treatments that differ significantly from the untreated control are indicated in red and the treatments that do not differ significantly from untreated control are indicated in blue. Boxplots with the same letters do not differ significantly at $P = 0.05$

reduce the number of live *Candidatus* Liberibacter *asiaticus* (Citrus Huanglongbing pathogen) cells (Trivedi et al. 2011). In our study, oak–bark compost enhanced plant resistance to *P. infestans* in whole plant and detached leaf assays. This may be linked to the activation of ISR or the production of metabolites by endophytes present in the compost. Another factor that can contribute in the success of plant protection is the use of a large volume of composts (> 15%) (Noble and Coventry 2005; Bahramisharif et al. 2013), due to an increase in microbial community. In our study, we used a 25% (v/v) of oak–bark compost.

Plant growth and protection were also present in treatments with *B. subtilis* subsp. *subtilis.* This Gram-positive bacterial strain is one of the major PGPRs that has shown promise for increasing crop yields. A number of mechanisms for plant growth promotion and pathogen protection have been proposed for *B. subtilis* subsp. *subtilis,* including the production of phytohormones, delivery of nutrients and stimulation of the ISR (Beneduzi et al. 2012; Pieterse et al. 2014). In prior studies on tomato seedlings, *B. subtilis* has been shown to be effective in promoting growth which may be due to an increase in plant hormone production such as indole-3-acetic acid (IAA) and gibberellic acid (GA_3) (Chowdappa et al. 2013; Cendales et al. 2017). In our study, plants treated with *B. subtilis* subsp. *subtilis* were 2.5-fold larger in biomass. Furthermore, the growth promotion was significantly enhanced when *B. subtilis* subsp. *subtilis* was combined with the compost. A recent study by Rao et al. (2017) found that *B. subtilis* enriched vermicompost treatment increase carrot (*Daucus carota* subsp. *sativus* Hoffm.) yields by 28.8%.

Likewise, *B. subtilis* proved to be effective for enhancing plant resistance under biotic stress. In our study, tomato plants treated with *B. subtilis* were resistant to the pathogen not only in the whole plant assays, but also in detached leaf assays. This indicates that the beneficial effect was most likely through a systemic plant response, because *B. subtilis* was not detected in the detached leaf tissue, where the pathogen challenge took place. Plant resistance was further enhanced by the application of *B. subtilis* subsp. *subtilis* enriched oak–bark compost in both whole plant and detached leaf assays. This may be due to the improvement in root colonization by the bio-agent, as compost provides additional nutrition for the bacteria. The application of combined *B. subtilis* and vermicompost has been shown to be effective in the reduction of nematode population and soft rot disease incidence in carrot (Rao et al. 2017). The combination of composts and biological agents has been reported for a few soilborne pathogens and *P. capsici* on several crops including beans (*Phaseolus vulgaris* L.), onion (*Allium cepa* L.), pepper (*Capsicum annuum* L.), rooibos (*Aspalathus linearis* (N.L. Burm.) R. Dahlgr.) and turf grass (Nakasaki et al. 1998; Chae et al. 2006; Coventry et al. 2006; Pugliese et al. 2011; Bahramisharif et al. 2013). The co-inoculation of compost with *B. subtilis* subsp. *subtilis* could enhance the consistency in disease suppression, as also reviewed previously (Noble 2011). Here, anthocyanin accumulation in leaflet of plants treated with either *B. subtilis* subsp. *subtilis* or in combination with the compost was not enhanced. Anthocyanins are commonly upregulated in response to plant stress caused by biotic or abiotic factors (Dixon and Paiva 1995; de Vries et al. 2018). However, similar to this study, Yoon et al. (2015) found a reduction in the total anthocyanin content after black rice bran was fermented with *B. subtilis.* Likewise, the anthocyanin level was decreased in *B. subtilis* fermented pigeon pea (Lee et al. 2015). This may be due to the hydrolysis of anthocyanin glycosides by β-glucosidase produced by *B. subtilis* (Asha et al. 2015).

Treatment with *Trichoderma harzianum* stimulated plant growth and protected plants from *P. infestans*, but only in whole plant assays. The plant protection by *T. harzianum* was not as great as when plants were treated with *B. subtilis* subsp. *subtilis*, in both whole plant and detached leaf assays. In contrast, *T. harzianum* has been reported to have greater potential for suppressing late blight disease incidence and severity in potato than *B. subtilis* (Wharton et al. 2012). The mode of action for growth promotion and plant protection by *T. harzianum* in whole plant assays may involve mycoparasitism, antibiotic production, competition, enzyme biosynthesis or ISR (Howell 2003). In this study, the level

of anthocyanin was elevated in treatments that included *T. harzianum*. In other studies, plants exposed to volatiles of *Trichoderma* showed elevated levels of anthocyanin levels and were more resistant to *Botrytis cinerea* and *Alternaria brassicicola* (Schwein.) Wiltshire (Kottb et al. 2015). The application of *T. harzianum* in combination with oak–bark compost negatively affected the root growth and resulted in significantly higher disease severity in both whole plant and detached leaf assays. Likewise, on other crops, the co-inoculation of *T. harzianum* with compost was shown to be ineffective at suppressing *P. ultimum* in cucumber and *Phytophthora nicotianae* Breda de Haan in tomato (Pugliese et al. 2011). One hypothesis for the negative impact in the combined treatment is that *T. harzianum* competes with or acts antagonistically on the beneficial members of the microbial community in the compost, leading to an increase in disease severity. On the other hand, the co-inoculation of *T. harzianum* and compost has been reported to improve saline soil quality (Mbarki et al. 2017). In the current study, it was notable that the negative impact of the combined *T. harzianum* and compost treatment could be nullified if *B. subtilis* subsp. *subtilis* was also added, suggesting that this bacterial agent is insensitive to *T. harzianum*.

Variation in tomato growth and protection was observed for the two commercial products, FZB24 and FZB42. In general, FZB24 positively impacted growth more than FZB42. Previously, Gül et al. (2008) showed that the two strains of *B. amyloliquefaciens*, FZB24 and FZB42, increased tomato yield by nearly 10% in the open hydroponic system; but, they both did not affect plant growth in the closed hydroponic system. Tryptophan-dependent synthesis of IAA has been implicated for the stimulation of plant growth by *B. amyloliquefaciens* (Idris et al. 2004, 2007). The FZB24 product was also better at plant protection than FZB42 in whole plant assays. However, none of these products were able to suppress late blight in detached leaf assays, which may be explained by the failure of these products to induce systemic defense in the host. The use of FZB24 has been reported to be effective in reducing disease caused by the oomycete *Pythium aphanidermatum* (Edson) Fitzp. at early stages in a hydroponic system of tomato (Grosch et al. 1999). In tomato plants, FZB24 product has also been shown to be effective against Fusarium crown and root rot (Myresiotis et al. 2012). Furthermore, FZB24 was tested against *Tilletia tritici* (Bjerk.) G. Winter, the causal agent of common bunt in wheat, and showed some beneficial activity under controlled conditions, but not in the field (Koch et al. 2006). The FZB42 product has been found to have antifungal activity towards *Fusarium graminearum* Schwabe which causes Fusarium head blight in cereal crops (Gu et al. 2017). The mechanisms involved in disease suppression by these products may be related to the enhancement of plant physical status through expression and upregulation of plant's defense compounds and genes, leading to plant stress-resistance, disease-resistance and growth (Xie et al. 2017). In the current study, FZB24 and FZB42 significantly increased anthocyanin content in tomato plants. The use of *Bacillus amyloliquefaciens* has been shown to significantly improve growth, yield and quality of strawberry fruits due to an increase in the level of natural antioxidants such as anthocyanins (Rahman et al. 2018). Other factors that can possibly be associated with disease suppression are the production of antifungal compounds such as Bacillomycin D (Gu et al. 2017) or the secretion of proteins such as acetolactate synthase (AlsS) by *B. amyloliquefaciens* that elicit plant innate immunity (Kierul et al. 2015). The co-inoculation of oak–bark compost and the commercial products resulted in an increase in plant growth but did not enhance plant protection further, compared to each product alone. It was also notable that compared to the untreated control, compost significantly decreased the level of anthocyanin when combined with the commercial products.

In conclusion, this study showed that oak–bark compost as a standalone treatment or in combination with *B. subtilis* subsp. *subtilis* and commercial products can be effective in enhancing tomato growth and resistance under biotic stress conditions. The combination of the compost and *B. subtilis* subsp. *subtilis* showed the greatest promise for obtaining better growth and more effective and consistent plant protection, although this did not correspond to higher levels of anthocyanin in leaves. Future research should determine the mechanistic basis for plant growth promotion and protection in the combination treatment of oak–bark compost with *B. subtilis* subsp. *subtilis.*

Author contribution statement AB and LER conceived and designed research. AB conducted experiments and analyzed data. AB wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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