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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.*

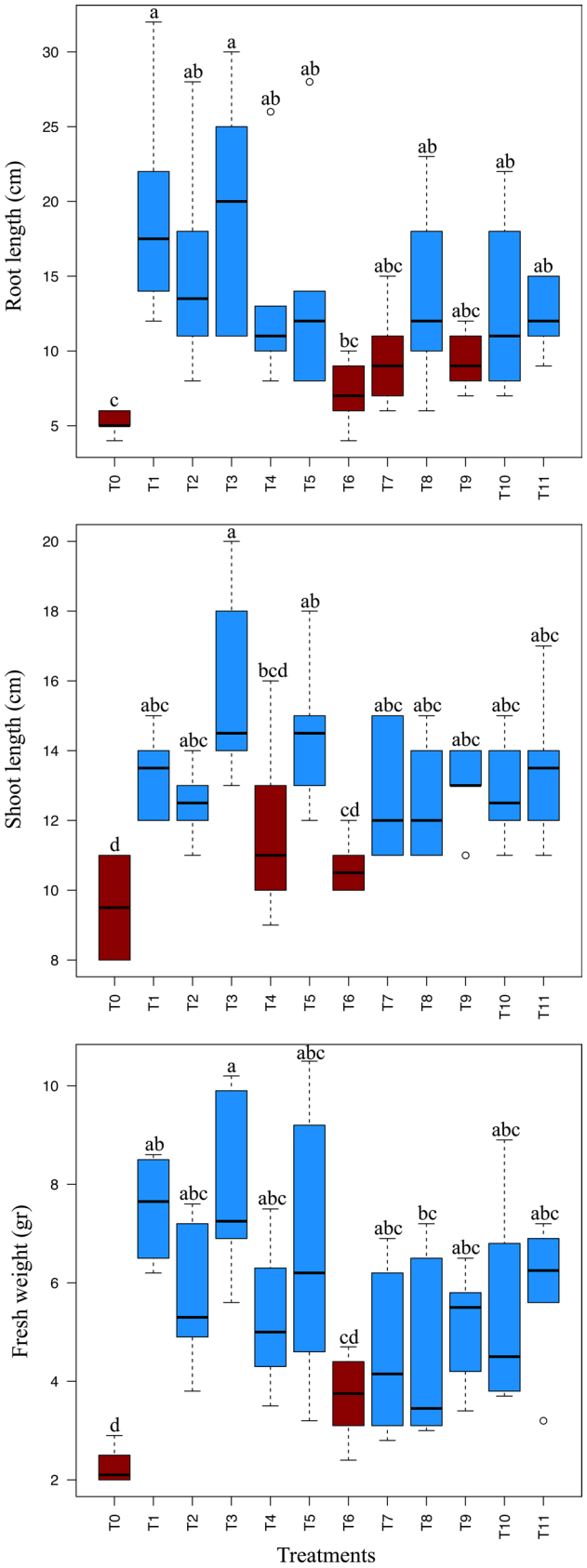

Fig. 1 Effect of the 12 treatments on root length, shoot length and fresh weight of tomato plants. The treatments were as follows: T0 (untreated control), T1 (compost), T2 (*B. subtilis* subsp. *subtilis*), T3 (compost + *B. subtlis* subsp. *subtilis),* T4 (FZB24), T5 (compost + FZB24), T6 (FZB42), T7 (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.

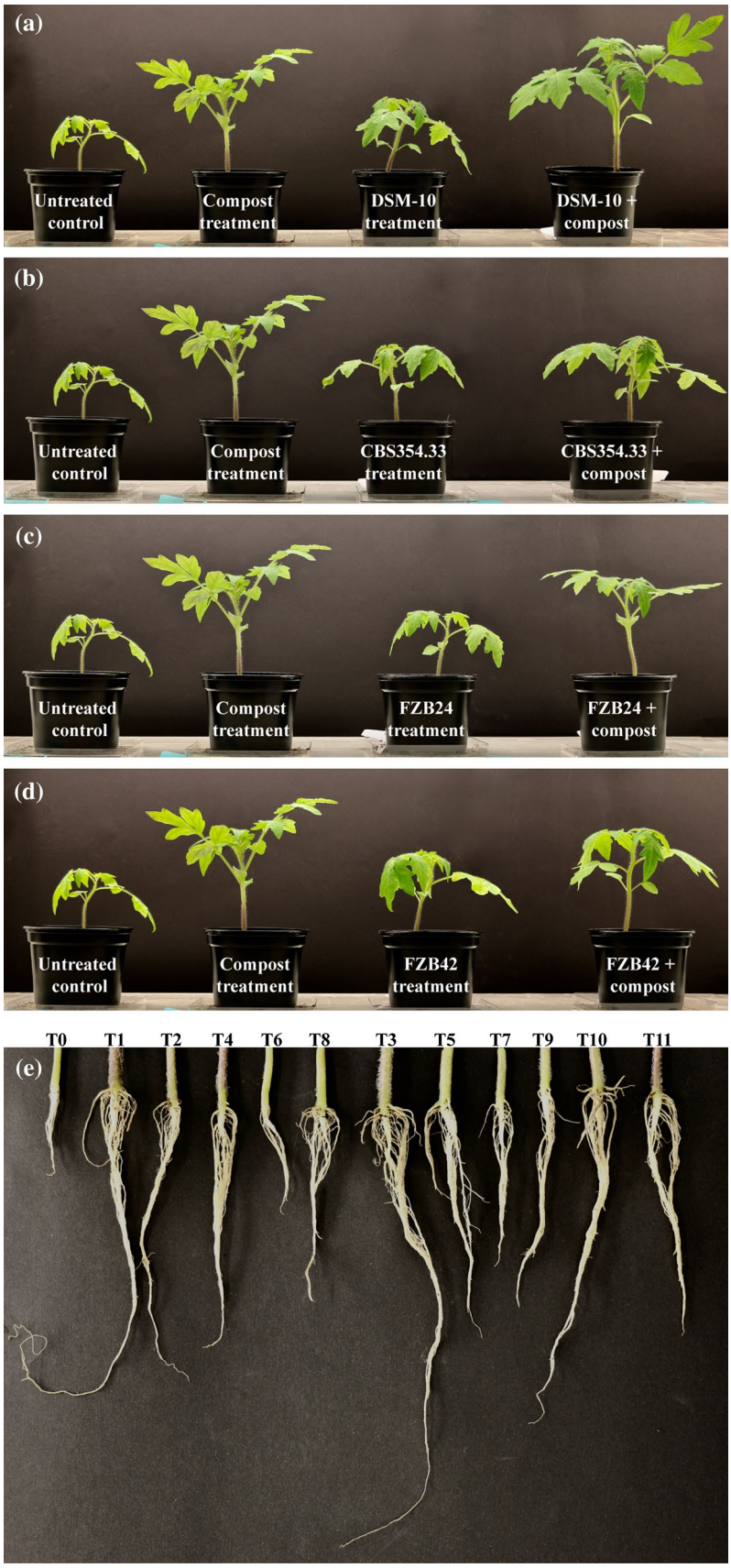

Fig. 2 Effect on tomato growth of treatments with *B. subtlis* subsp. *subtilis* (DSM-10, **a**); *T. harzianum* (CBS354.33, **b**); FZB24 (**c**) and FZB42 (**d**). **e** Effect of the 12 treatments on root growth

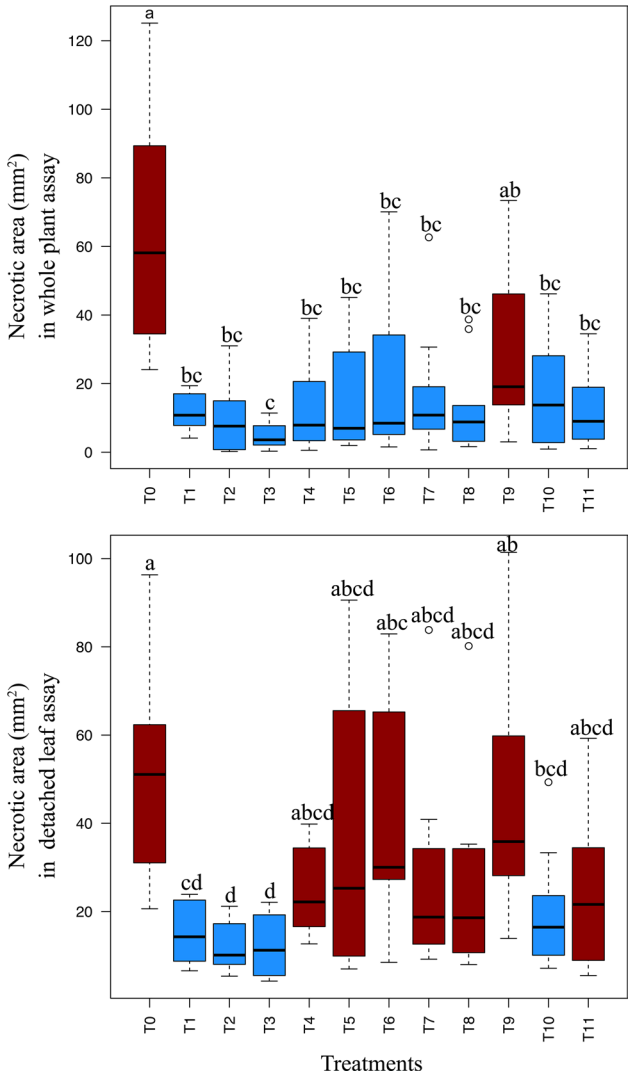

Fig. 3 Necrotic area for 12 treatments (T0–T11) in the whole plant and detached leaf assays. Data are from five replicates over two trials. The treatments that differ significantly from the untreated control are indicated in blue and the treatments that do not differ significantly from untreated control 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

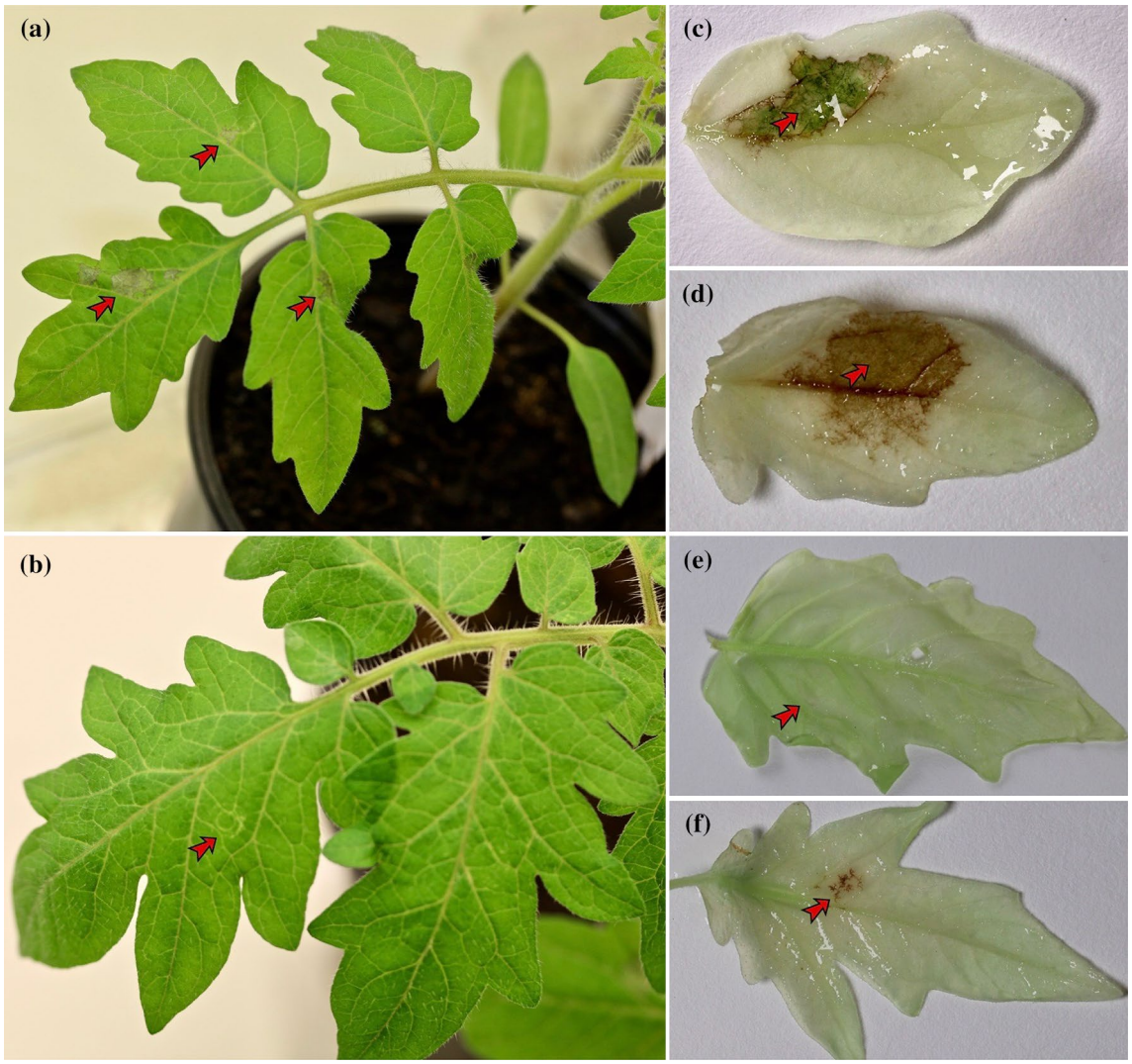

Fig. 4 Symptoms of 4-week-old Moneymaker tomato plants from infection by *P. infestans* isolate, D12-2. The red arrows indicate the location of infiltration/loading of *P. infestans* zoospores. **a** The tomato seed planted in un-amended soil. **b** The seed planted in oak–bark-compost-amended soil, 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

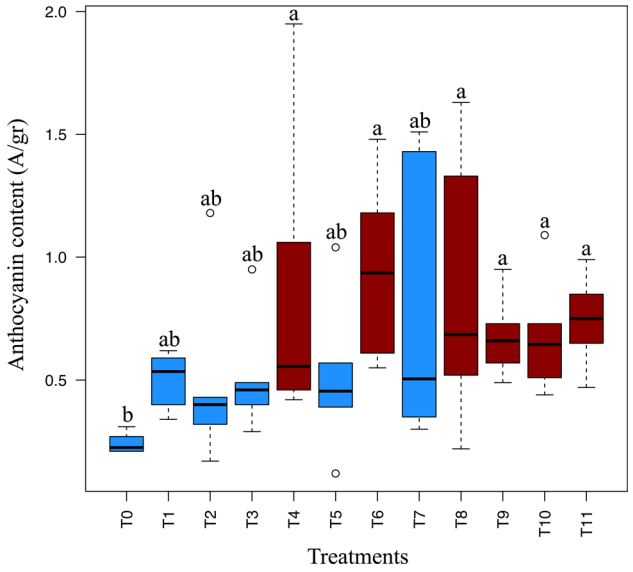

Fig. 5 Effect of the 12 treatments on anthocyanin accumulation in tomato leaflets. Six biological replicates were analyzed for each treatment. Anthocyanins (A) were evaluated by measuring the absorbance at 530 and 657 nm. The treatments that differ significantly from the untreated control are indicated 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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