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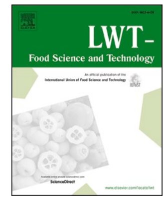

Biocontrol potential of lipopeptides produced by the novel *Bacillus altitudinis* strain TM22A against postharvest Alternaria rot of tomato

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A R T I C L E I N F O

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A B S T R A C T

Bacillus genus exhibits antagonistic potential against a broad range of pathogenic fungi. This study presents the antifungal potential of lipopeptides (LPs) produced by a novel strain *Bacillus altitudinis* TM22A to suppress the infection of Alternaria rot on tomato. We investigated the antifungal effect of ten *Bacillus* species against *Alternaria alternata*, where four *Bacillus* strains TM22A, MCM61, S2, and CPCF54 exhibited antifungal activity. The LPs extracted from TM22A, MCM61, S2, and CPCF54 restricted mycelial growth and conidial germination of *A. alternata in vitro* with the highest inhibition produced by TM22A. Liquid chromatography and mass spectrometry (LC-MS) analysis revealed the presence of surfactin, fengycin, iturin, bacillibactin, and bacilysin in strain TM22A at 1036.69, 1435.76, 1085.58, 883.26, and 271.13 m/z respectively. In an *In planta* assay LPs from TM22A caused the lowest lesion diameter and disease incidence on tomato fruit. Furthermore, the activity of defense enzymes viz, catalase (CAT), polyphenol oxidase (PPO), peroxidase (POD), superoxide dismutase (SOD), and phenylalanine ammonia-lyase (PAL) was enhanced in *Bacillus* LPs treated tomato fruit with the more pronounced impact by TM22A. Postharvest quality analysis revealed little impact on ascorbic acid, fruit pH, total soluble solids (TSS), titratable acidity (TA), weight loss, and fruit firmness compared to healthy control. This is the first comprehensive study to reveal the antifungal effect of *Bacillus*-derived LPs against postharvest Alternaria rot of tomato and its role in defense elicitation to protect the fruit from pathogen invasion.

1. Introduction

Tomato (*Solanum lycopersicum* L.) is a vital horticultural crop all over the world but it is susceptible to different postharvest decay-associated fungi (Singh, Prasad, & Singh, 2017). There are many biotic and abiotic factors like pests, pathogens, salt stress, heat stress, drought stress, and inadequate handling of the fruit that exert a negative impact on the postharvest quality of tomato and other horticultural crops (Steinberg et al., 2020). Moreover, inadequate storage facility, transportation, processing, packaging, and marketing are the major events responsible for postharvest losses of fruit.

Several fungal pathogens, such as *Alternaria alternata, Geotrichum candidum, Phoma* spp., *Botrytis cinerea, Didymella lycopersici, Rhizopus stolonifer*, and *Fusarium acuminatum* are associated with postharvest rots of tomato (Petrasch et al., 2019). Temperature and humditity are considered the most critical environmental factors for the development

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of postharvest fungal infections in fruit (You, Zhou, Duan, Mao, & Li, 2022). *A. alternata* is a threatening postharvest pathogen among filamentous fungi linked with tomato fruit rots causing a significant economic loss (Slathia et al., 2021). Additionally, when there are lesions on the fruit surface, *A. alternata* becomes aggressive, and it gets debilitated when stored for an extended period. *Alternaria* develops smooth, dark brown to black, somewhat sunken, firm lesions that can grow up to several centimeters in diameter (Yang et al., 2020).

Alternaria species require low temperature for their growth and development, therefore, they cause spoilage of fruit and vegetables during cold storage conditions (Ostry, 2008). The control of postharvest pathogens has been achieved in several ways (Romanazzi et al., 2016) such as chemical additives, physical therapies, UV-C irradiations, chemical and biological control. Recently, eugenol and citral essential oils have been used to treat postharvest fungal infections (Ju, Guo, Cheng, & Yaoc, 2022) and the applications of essential oils as food preservatives have been elucidated (Wang, Gao, Zhao, & Ju, 2023; Wang, Zhao, et al., 2023; Wu, Zhao, Li, Huang, & Ju, 2022). In view of the health and environmental safety concerns, the use of synthetic chemicals is restricted. Instead of the chemical fungicides, the use of sustainable biological strategies such as plant extracts, essential oils, and antagonistic microbial agents has been employed to decrease the intensity of postharvest fruit and vegetable rots (Ahmet, Saban, Hamdullah, & Ercan, 2005; Moosa, Zulfiqar, & Siddique, 2022).

Therefore, the use of biological control agents as a sustainable strategy for controlling the postharvest diseases is an emerging field of research. In this regard, several antagonistic microbial agents have been explored for their inhibitory potential against plant pathogens. *Bacillus* is a very efficient biological control agent to suppress plant pathogens (Farzand et al., 2019a; b; Fatima et al., 2023). The suppressing ability of *Bacillus* species is linked with the production of a wide range of antimicrobial chemicals including hydrolytic enzymes, antibiotics, lipopeptides (LPs), and volatile metabolites (Farzand et al., 2019a; b; Farzand et al., 2020).

Lipopeptides are the fatty acid chains linked with oligopeptides. They are classified into three main families including surfactin, fengycin, and iturin (Ongena & Jacques, 2008). All these families possess antifungal ptoential and are considered alternative way to control fungal pathogens (Farzand et al., 2019a; b). The LPs extracted from *Bacillus* strains have shown excellent antifungal activity against several decay-associated fungi (Alvarez et al., 2012; Farzand et al., 2019a; b). The detection of LPs is critical for determining *Bacillus* species with antagonistic potential. The actual LPs produced by *Bacillus* strains can be detected using liquid chromatography-mass spectrometry (LC-MS) which is a high throughput, more accurate and sensitive method of detection (Farzand et al., 2019a).

The LPs have also been associated with the activation of plant natural defense enzymes (Farzand et al., 2019b). Plants defend themselves against biotic and abiotic stresses by utilizing their defense system comprising of enzymatic and non-enzymatic enzymes (Moosa, Sahi, Khan, & Malik, 2019; Moosa et al., 2022). Disease control is usually acquired via triggering the defense enzyme acitivity. Different defense enzymes including PPO,SOD, POD, andCAT are important oxidative enzymes for maintaining cell structure via catalysis of lignin and other oxidative phenols (Avdiushko, Ye, & Kuc, 1993). The phenolic compounds are formed by another enzyme PAL and this enzyme plays a vital role to protect plants from several pathogens (Goy, Felix, Metraux, & Meins, 1992; Moosa et al., 2022). Several researchers have reported the defense role of these enzymes in protecting fresh produce against pathogens (Moosa et al., 2022; Sukorini, Sangchote, & Khewkhom, 2013).

We hypothesized that *Bacillus* LPs can reduce the development of *A. alternata* on tomato fruit by modulating the activity of defense enzymes such as POD, PAL, PPO, SOD and CAT. Therefore, the present study was designed to unravel the antifungal effect of *Bacillus* strains and LPs against Alternaria rot of tomato. Furthermore, to unravel the underlying defense mechanisms the impact of antifungal LPs on the defense enzymes and the postharvest quality of fruit was determined. To best of our knowledge, this is the first study reporting the the antifungal effect of *Bacillus* derived lipopeptides and their detailed mechanism of action to suppress postharvest Alternaria rot of tomato.

Table 1
List of *Bacillus* strains used for experimentation.

Sr No	*Bacillus* species	Strain code
1	*Bacillus subtilis*	MGRP21
2	*Bacillus atrophaeus*	MCM61
3	*Bacillus subtilis*	S2
4	*Bacillus subtilis*	CPCF54
5	*Bacillus altitudinis*	TM22A
6	*Bacillus altitudinis*	TM22
7	*Bacillus velezensis*	JM11
8	*Bacillus cereus*	CP1
9	*Bacillus amyloliquefaciens*	CFGP92
10	*Bacillus thuringiensis*	M3

2. Materials and methods

2.1. Fungal cultures Bacillus altitudinis strain TM22A

The pure culture of *A. alternata* was obtained from the fungal culture bank of the Department of Plant Pathology, The Islamia University of Bahawalpur, Pakistan. *A. alternata* isolate AAT-23 was isolated from infected tomato samples collected from local market of Bahawalpur, Pakistan. The culture was stored in 30 % glycerol solution at −80 °C. The culture was retrieved on Potato Dextrose Agar (PDA) medium and incubated at 25 ± 2 °C for 7 d.

2.2. Bacterial cultures

Bacillus strains were taken from the Molecular Plant Pathology Lab, Department of Plant Pathology, The Islamia University Bahawalpur, Pakistan (Table 1). These stock cultures of *Bacillus* strains were preserved at −80 °C in a 60 % glycerol solution. These cultures were recovered on Luria Bertani (LB) medium. Afterward, the *Bacillus* inoculated LB medium was incubated for 48 h at 37 °C for further use.

2.3. Fruit material

Healthy, disease-free, and fresh tomatoes cv. 'Rio Grande' were obtained from a fresh fruit and vegetable market at Bahawalpur, Pakistan. The fruit were used for the experiments on the same day.

2.4. In vitro direct antagonism assay

In a dual culture assay, the antifungal effect of *Bacillus* strains was investigated on the colony development of *A. alternata*. A culture block (5 mm) from a 7 d old fungal colony was positioned at the center of the PDA plates. Then, 10 μL bacterial culture (OD600 = 2.5) was poured on sterilized filter paper discs placed 3 cm away from the fungal culture block (Fatima et al., 2023). The plates were incubated at 25 ± 2 °C for 7 d. The inhibition zones around the fungal colony towards the edge of the plate were measured in cm. For the control treatment, PDA was inoculated with a 5 mm 7 d old culture block of the pathogen only. The experiment was executed three times with the same experimental settings having 6 replicates of each treatment in a completely randomized design.

2.5. Extraction of lipopeptides and antifungal assay with lipopeptides

The LPs were extracted from the best *Bacillus* strains with the highest inhibition and tested against the colony growth of *A. alternata*. To

extract the LPs, *Bacillus* strains were grown in a Landy medium (Sarwar et al., 2018). The flasks containing *Bacillus* inoculated Landy medium were incubated for 3 d at 30 °C using a rotary shaker at 180 *g*. Three days post-incubation *Bacillus* strains were centrifuged at 10,000 *g* for 15 min at 40 °C and cell-free supernatant was taken and incubated for 12 h at 4 °C and pH was maintained at 2 by adding 0.1 N HCl. Then it was again centrifuged at 10,000 *g* for 15 min. The obtained supernatant was discarded, and the pellet was set aside to dry. The dried pellet was dissolved in HPLC-grade methanol of neutral pH and filtered using 0.22 μm syringe filter paper to eliminate impurities. To test the antifungal activity of LPs isolated from *Bacillus* strains against *A. alternata*, a 7 d old fungal culture was inoculated at the center of a petri dish containing PDA medium. Later, 10 μL *Bacillus* LPs were inoculated on sterilized filter paper discs 3 cm away from the *A. alternata* culture. Then, the inhibition zones were measured in cm. This experiment was executed three times with six replicates of each treatment with the same experimental settings.

2.6. Liquid chromatography and mass spectrometry (LC-MS) analysis for the detection of lipopeptides

The LPs were extracted following the protocol described in section 2.5. To detect LPs extracted from *Bacillus* strains the system LC-MS surveyor (G2 QT of- XS, a waters) was used. UPLC C18 2.1 mm × 100 mm column containing ACQUITY UPLCBEH 1.7 m particles was employed. The volume of the injection was 2 μL with 0.4 mL min^{-1} flow rate. The components of the mobile phase were 1 = water, formic acid 0.1 %; 2 = acetonitrile. Formic acid 0.1 %; The LPs of the strains were eluted by the linear gradient: 5 % solution 1 for 2 min, and 95 % solution 2 for 2 min respectively. Mass spectrometry analysis was carried out by using an electrospray source in positive ion mode with MSE acquisition mode within the range of 50–1200 m/z. According to conditions, the LC-MS modalities were applied to ions set during analysis. The analysis was performed under certain conditions; V = 2.5 KV, T = 12 °C, E = 40 eV, where, V= Voltage source, T = Dissolution gas temperature, E = Collision energy. Masslynx software (Ver, 4.1) was used for data acquisition and processing.

2.7. Effect of lipopeptides on spore germination

The hanging drop method was used to test the effect of LPs on spore germination (Ju et al., 2020). *A. alternata* was grown on PDA medium and incubated at 25 ± 2 °C for 7 d. The fungal spores were washed and diluted with sterile distilled water and around 40 spores per view were observed under a microscope at 40× magnification power. The fungal spore suspension was mixed with LPs solution in an equal volume and then 10 μL of this suspension was dropped on a clean glass slide. The fungal spore suspension was combined with an equivalent volume of sterile distilled water for the control treatment and both suspensions were cultured for 8 h at 28 °C. The spore morphology was observed under a microscope and the number of total spores and germinated spores was counted in each view. The germination of conidia was recorded in percentage (%) when the length of the germ tube was larger than the spore diameter. The following formula was used for the measurement of spore germination; Spore germination rate (%) = Number of spores germinated/Total number of spores × 100. Spore germination was counted in six replicates of each treatment where six Petri plates containing fungal culture were considered as three replicates. The experiment was repeated twice under the same experimental conditions.

2.8. In Planta experiment

The LPs extracted from *Bacillus* strains were employed to control *A. alternata* infection on tomatoes. Healthy, damage-free, mature tomato fruit were rinsed with sterilized distilled water, surface disinfested with 1 % NaClO for 1 min and then washed with sterilized distilled water. The tomato fruit were wounded with the help of a sterile needle and 10 μL *Bacillus* LPs were deposited into the wounds with the help of a micropipette and later 10 μL of fungal conidial suspension was inoculated in the same wounds with the help of a micropipette. The fruit subjected to treatment were kept in autoclaved plastic boxes and incubated at 25 ± 2 °C for 7 d. Lesion diameter and disease incidence were assessed 7 d post-incubation. Lesion diameter was measured in cm by using a Vernier caliper. Each treatment was replicated six times and the experiment contained 36 tomato fruit. The experiment was repeated twice under the same experimental settings. The following formula was used to calculate disease incidence (%) on treated fruit; Disease incidence (%) = Number of infected fruit/Number of total fruit × 100.

2.9. Enzyme activity assay

2.9.1. Extraction of sample

The tomato fruit segment was excised from the outer pericarp, 0.5 g was weighed on an electric weight balance and the sample was placed on a pre-cooled mortar. The fruit segment was ground into 3 mL phosphate buffer in a pestle mortar and centrifuged at 13,000 *g* at 4 °C for 20 min and stored at 4 °C in separate tubes. The same procedure was repeated for all the samples. The total protein content of the extract was measured using the Bradford technique (Bradford, 1976), utilizing bovine albumin serum as a reference. The activity of each enzyme was determined at three different time intervals 0 h, 48 h, and 96 h. For each experiment 30 treated tomato fruit were used.

2.9.2. Peroxidase assay

The POD enzyme activity was evaluated by using the method given by Meena et al. (2023), with some modifications. The reaction solution consisted of 23 μL of 50 mM potassium phosphate buffer of pH 7, 50 μL of 20 mM guaiacol solution, 30 μL of 12 mM H_2O_2, and 70 μL enzyme extract respectively. The absorbance was measured using a spectrophotometer at 436 nm. The measurement of POD activity was carried out in U mg^{-1} protein. The enzyme activity was determined in five replicates (tomato fruit) and the experiment was repeated twice under the same experimental settings.

2.9.3. Polyphenol oxidase assay

The PPO enzyme activity was evaluated by using the method of Vanama et al. (2023), with a few modifications. The reaction solution contained 1 mL of 0.1 M potassium phosphate buffer of pH 6.0, 0.5 mL of 0.1 M catechol, and 0.25 mL enzyme extract. After 2 min 0.5 mL H_2SO_4 was added to stop the reaction and the absorbance was measured at 495 nm on a spectrophotometer. PPO activity was expressed as units of enzyme activity unit mg^{-1} protein. The enzyme activity was measured in five replicates (tomato fruit) and the experiment was repeated twice under the same conditions.

2.9.4. Phenylalanine ammonia-lyase assay

To measure the PAL activity the treated fruit were peeled, and 5 g of fruit tissue was crushed into a pre-cooled 50 mL acetone solution. The obtained filtrate was homogenized using a Buchner funnel. The residual was rinsed with chilled acetone and dried at room temperature. The PAL enzyme reaction solution contained 0.05 g of acetone powder, 100 mM sodium borate buffer (1.5 mL) pH 8.0, and 20 mM β-mercaptoethanol. The whole supernatant was purified with 60 % ammonium sulfate by using salting out proteins and the pure extract was desalted using SephadexTM G-50 column. The pure extract was then recovered in 1 mL of 100 mM sodium borate buffer (pH 8.0) (Moosa et al., 2022). The enzyme activity was determined by measuring the absorbance at 290 nm of cinnamic acid at 40 °C for 2 h. Partially purified enzyme 0.3 mL and 0.1 mL L-phenylalanine respectively. The data was calculated from five replicates of each treatment and the experiment was repeated twice. The enzyme activity was presented as unit mg^{-1} protein.

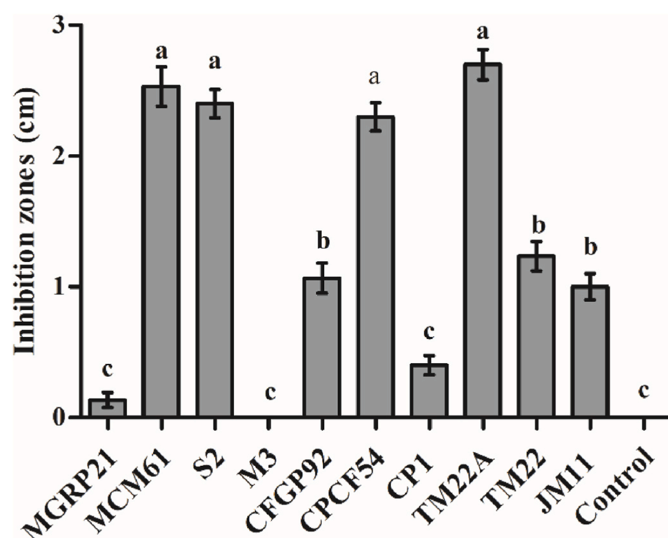

Fig. 1. Effect of *Bacillus* strains on the colony growth inhibition of *A. alternata*. Values are the mean of six replicates and the alphabets over the column indicate that the means with the same alphabets are not different from each other at $P = 0.05$ analyzed by Tukey's HSD test. Error bars over the column represent the standard error ± SE of means.

2.9.5. Superoxide dismutase assay

To determine SOD activity the reaction mixture included potassium phosphate buffer 50 mM of pH 7.8, methionine 13 mM, riboflavin 2 μL, EDTA 0.1 mM, 75 μM nitroblue tetrazolium, and 20 μM enzyme extract. Then, riboflavin was added to the solution at the end of the reaction, the tubes were stirred and placed under 18 W fluorescent lamps to initiate the reaction. After 10 min, the reaction was halted by turning off the light. The tubes without illumination under fluorescent lamps were kept as a blank treatment. The reaction solution was shaken in the tubes before putting into 1.5 mL cuvettes. The total SOD activity was measured spectrophotometrically at 560 nm by calculating the photochemical reduction of nitro blue tetrazolium (NBT) at 50 % inhibition (Catiempo, Photchanachai, Bayogan, & Vichitsoonthonkul, 2021). The measurement of SOD activity was carried out in U mg^{-1} protein. SOD activity was measured in five replicates (tomato fruit) and the experiment was repeated twice under the same experimental settings.

2.9.6. Catalase assay

To assess the CAT activity at 25 °C (Catiempo et al., 2021) the reaction mixture contained 40 mM H_2O_2 in a 50 mM phosphate buffer (pH 7.0), and 0.1 mL of crude enzyme extract in a total volume of 3 mL. The CAT activity was measured at 240 nm on a spectrophotometer. The data was calculated from six replicates of each treatment. The measurement of CAT activity was carried out in Unit activity mg^{-1} protein. The enzyme activity was measured in five replicates (tomato fruit) and the experiment was repeated twice.

2.10. Evaluation of postharvest quality of tomato fruit

Fruit firmness was determined by using a penetrometer and expressed in unit N. Weight loss was calculated by using the following formula; Weight loss (%) = weight before applying the treatment – weight after treatment/weight before applying the treatment × 100. The TSS (%) of treated and untreated tomato fruit was checked by adding a few drops of fruit juice on a refractometer prism scale. Titratable acidity (%) was estimated by the titration method (Moosa et al., 2022). Ascorbic acid was determined by using the protocol of Klein and Perry (1982). The unit mg VIT C Kg^{-1} fresh weight of tomato was used to express the ascorbic acid content. The pH of tomato fruit juice was checked by using a pH meter. The data for all postharvest quality

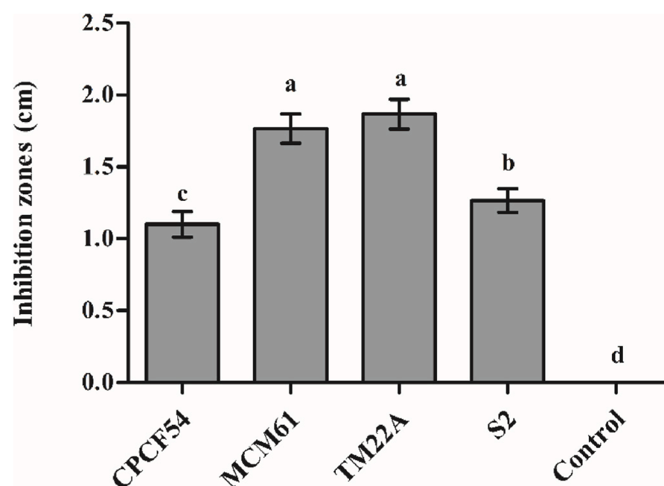

Fig. 2. Effect of lipopeptides (LPs) extracted from *Bacillus* strains on the colony growth inhibition of *A. alternata*. Values are mean of six replicates and the alphabets over the column indicate that the means with same alphabets are not different from each other at $P = 0.05$ analyzed by Tukey's HSD test. Error bars over the column represent the standard error ± SE of means.

parameters was calculated from six replicates of each treatment and the experiment was repeated twice under the same conditions.

2.11. Statistical analysis

The experimental design for all experiments conducted in this study was a completely randomized design (CRD). Tukey's HSD at P = 0.05 was used to examine the differences between treatments after the data were subjected to one-way ANOVA using Statistix statistical package (ver. 10). Graph Pad Prism software (ver. 5) was used for the designing of graphs.

3. Results and discussion

3.1. In vitro antagonism assay with Bacillus strains

Among ten *Bacillus* strains nine strains exhibited inhibition with a varying degree but M3 failed to produce inhibition of *A. alternata*. The highest inhibition was showed by TM22A with an inhibition zone of 2.7 cm followed by MCM61 with an inhibition zone of 2.5 cm (Fig. 1). The lowest inhibition was produced by CP1 (0.4 cm) and MGRP21 (0.1 cm) compared to the control. The inhibition was zero in the control group because no *Bacillus* treatment was applied to suppress *A. alternata*. Four *Bacillus* strains TM22A, MCM61, S2, and CPCF54 gave good suppression of *A. alternata*. Endophytic *Bacillus* species have been frequently employed as biocontrol agents to combat fungal infections. Several researchers have previously reported the inhibitory potential of *Bacillus* strains against various plant pathogenic fungi in direct antagonism assays (Farzand, Moosa, Zubair, Khan, Hanif, et al., 2019; Fatima et al., 2023). The studies of (Gao, Zhang, Liu, Han, & Zhang, 2017; Lim et al., 2017), support our work, where the simultaneous release of volatile and diffusible organic compounds to the culture medium was attributed to being the cause of the maximum inhibition in the direct antagonism test.

3.2. In vitro antifungal assay with Bacillus LPs

The LPs extracted from four antifungal *Bacillus* strains produced inhibition zones with varying degrees. The greatest inhibition zone was shown by TM22A (1.86 cm) followed by MCM61 with an inhibition zone of 1.76 cm. The lowest inhibition zone was produced by CPCF54 (1.1 cm) as compared to the control (Fig. 2). In the control group only *A. alternata* was inoculated at the center of the PDA plate hence no

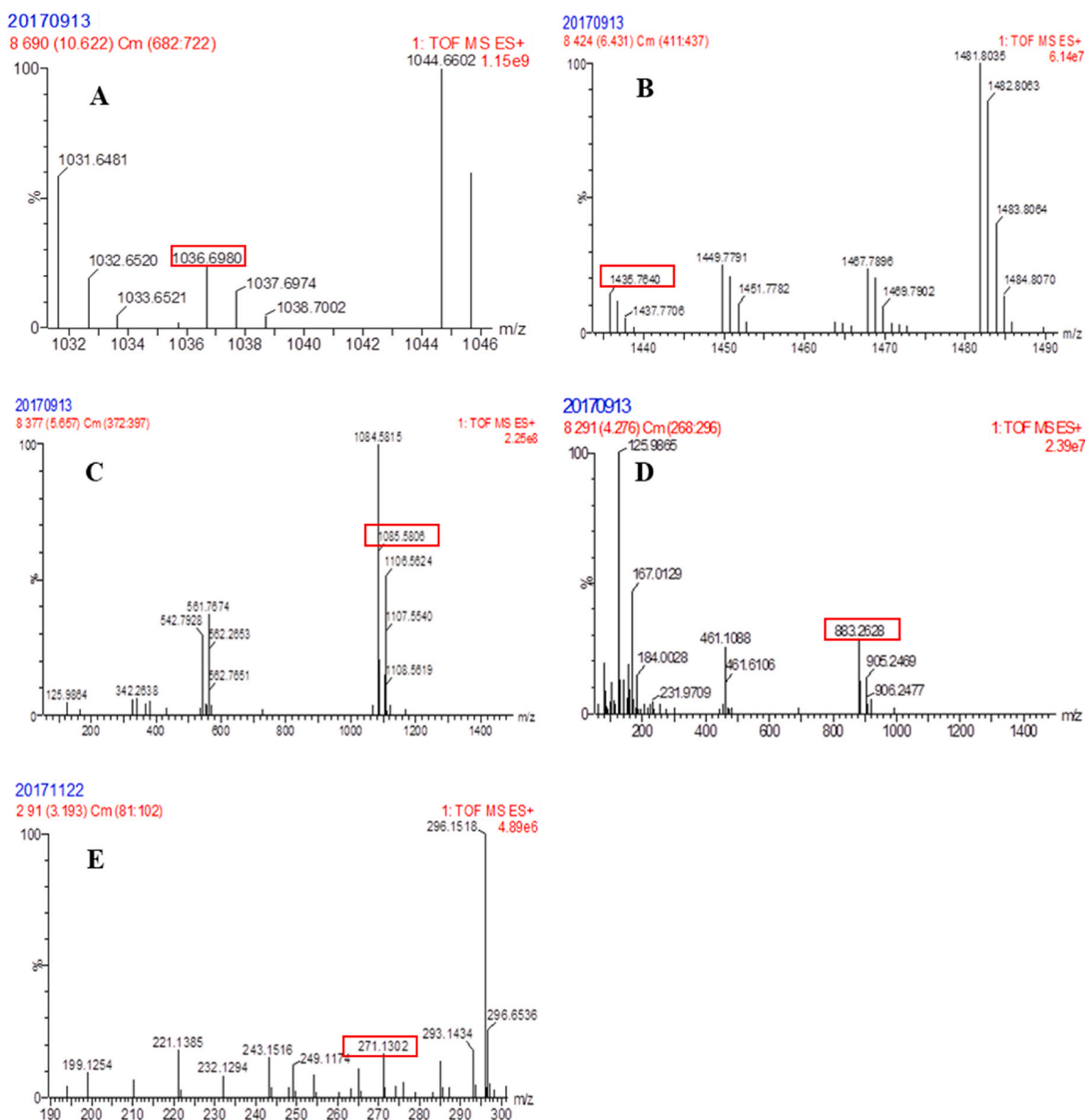

Fig. 3. LC-MS chromatogram of five antimicrobial compounds in *B. altitudinis* (TM22A). A; Surfactin 1036.6980 m/z, B; Fengycin 1435.7640 m/z, C; Iturin 1085.5806 m/z, D; Bacillibactin 883.2628 m/z, and E; Bacilysin 271.1303 m/z.

inhibition zone was observed because *Bacillus* LPs were not applied. In support of our work, the antifungal potential of *Bacillus* LPs has been previously reported (Farzand et al., 2019a; b). These lipopeptides are safe for consumption with no toxic effects on human health and their antifungal effect has been reported in several previous reports (Cozzolino et al., 2020). Lipopeptides are biomolecules that are nontoxic, biodegradable, very stable, environmentally safe, and nonpolluting (Farzand, Moosa, Zubair, Khan, Hanif et al., 2019 a,b). Fengycin possesses antifungal activity against several filamentous fungi (Guo et al., 2014). Fengycin interacts with fungal cell membrane phospholipid and sterol molecules and alters the cell membrane structure and permeability (Deleu, Paquot, & Nylander, 2005). Fengycin-type LPs generated by *B. subtilis* NCD-2 were the primary cause of antifungal activity against *Rhizoctonia solani* and prevented damping off disease of cotton (Guo et al., 2014).

It has been also reported that iturin proved effective against phytopathogens, through direct antifungal activity (Farzand et al., 2020). In another instance, the lipopeptide iturin A extracted from *B. subtilis* showed antifungal activity against *Colletotrichum gloeosporioides* (Kim, Ryu, Kim, & Chi, 2010), and iturin A, and bacillomycin extracted from *B. subtilis* were inhibitory against *Podosphaera fusca* (Romero et al., 2007). Iturin can bind with biological membranes, settles into lipid bilayers, creates irreversible pores and the surfactin molecules enter through these pores and promote the breakdown of membrane and solubilization (Ongena & Jacques, 2008). According to several previous reports fengycin, iturin, and bacillomycin D showed antifungal activity which was related to the accumulation of ROS and eventually induced the death of fungal cells (Gu et al., 2017; Tang et al., 2014).

3.3. Detection of lipopeptides by LCMS

The LPs were detected from the best *Bacillus* strain TM22A showing higher inhibition in direct antagonism and lipopeptide assay. In LCMS analysis several peaks for various LPs were found between 200 and 1500 m/z. These lipopeptides were putatively designated based on m/z values. LCMS analysis revealed the strain TM22A possessed the ability to

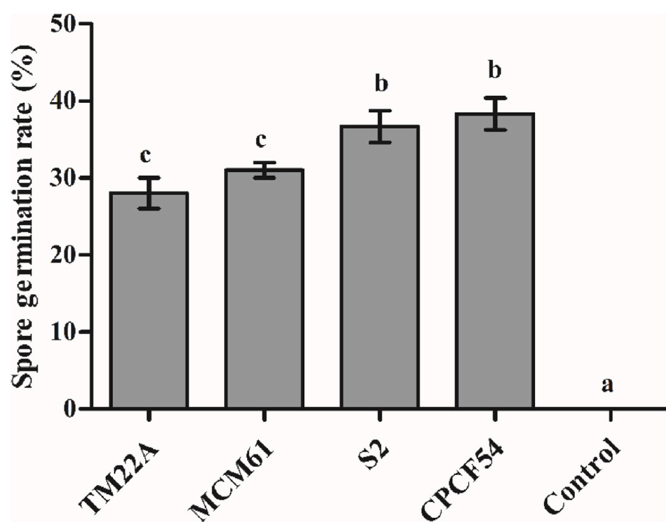

Fig. 4. Effects of lipopeptides (LPs) produced by *Bacillus* on the spore germination of *A. alternata*.

produce five different LPs including surfactin, fengycin, iturin, bacilysin, and bacillibactin. The three main families of lipopeptides surfactin, fengycin, and iturin, were detected at 1036.69 (Fig. 3A), 1435.76 (Fig. 3B), and 1085.58 m/z (Fig. 3C) respectively. The peaks for bacillibactin and bacilysin were observed at 883.26 (Fig. 3D) and 271.13 m/z (Fig. 3E). These peaks were assigned by comparing the findings of this investigation with lipopeptides already identified in the literature. Previous studies have reported a direct link between the simultaneous detection and synthesis of antifungal LPs and their antifungal activity (Um, Fraimout, Sapountzis, Oh, & Poulsen, 2013). The peaks for detected LPs were validated by comparing with previous reports where similar peaks (m/z) were reported for surfactin, fengycin, iturin, bacillibactin, and bacilysin (Farzand et al., 2019a). Therefore, the antifungal activity of *Bacillus* species in our study can be attributed to the production of antifungal LPs.

3.4. Effect of lipopeptides on spore germination

The effect of LPs extracted from four *Bacillus* strains was assessed on the spore germination of *A. alternata*. All strains showed inhibitory potential against spore germination of *A. alternata* but with a varying degree. The lowest rate 28 % of spore germination was showed by TM22A followed by MCM61 with a spore germination rate of 31 % relative to control (Fig. 4). LPs extracted from four *Bacillus* strains also inhibited the spore germination of *A. alternata*. Similar to our work, fengycin has been reported to reduce spore production and germination of *Fusarium graminearum* (Gu et al., 2017). In another instance, mycelial growth and spore formation of *Botrytis cinerea* BC1301 were inhibited by the metabolites of *Bacillus velezensis* 5YN8 (Jiang et al., 2018). The LPs secreted by *B. subtilis* YM 10–20 may permeabilize the spores of fungus and reduce their germination rate (Chitarra et al., 2003). Therefore, the production of LPs can be a possible reason for the inhibition of fungal spore germination of *A. alternata* in our study.

3.5. In Planta assay

The effect of LPs extracted from four *Bacillus* strains exhibited a varying degree of disease incidence of *A. alternata* on tomato fruit. The lowest lesion diameter was produced by TM22A 0.17 cm followed by MCM61 with a lesion diameter of 0.25 cm relative to healthy control treatment. The highest lesion diameter (2.37 cm) was recorded in infected control treatment (Fig. 5A). The lowest disease incidence 16 % was showed by TM22A, followed by MCM61 with a disease incidence of

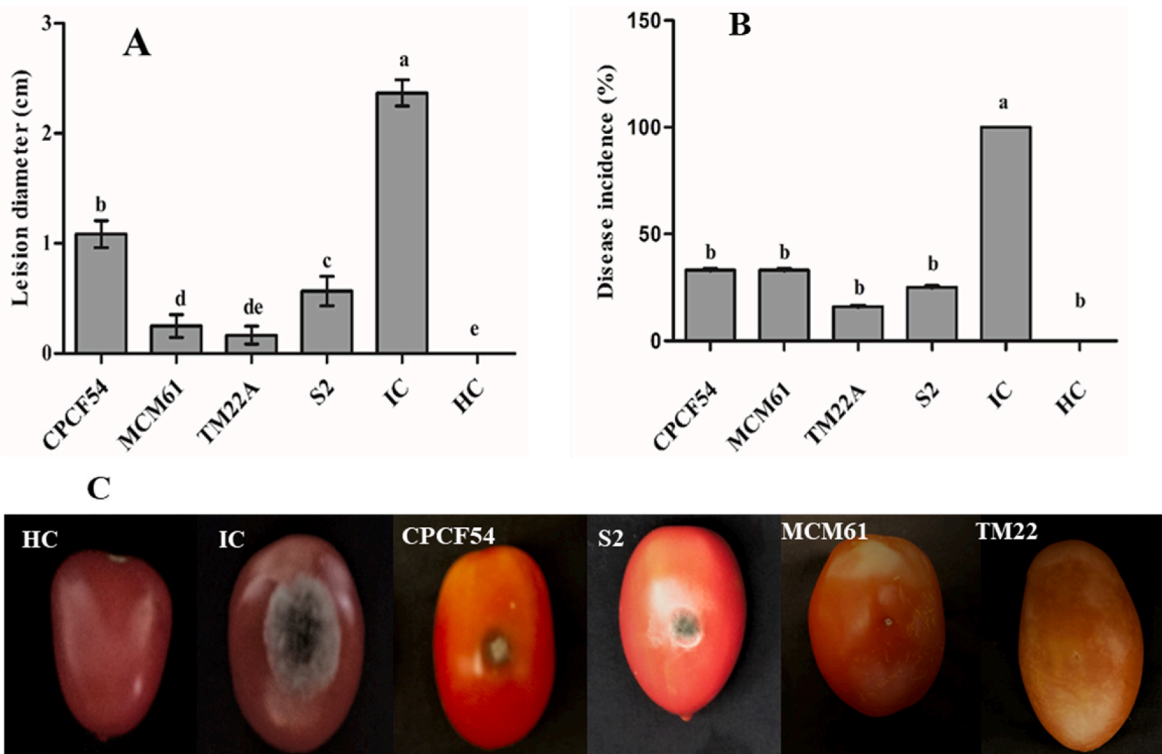

Fig. 5. Effect of lipopeptides (LPs) produced by *Bacillus* strains on A) lesion diameter and disease incidence of *A. alternata* on tomato fruit, B) Tomato fruit showing symptoms of Alternaria rot during *In Planta* assay. Values are mean of six replicates and the alphabets over the column indicate that the means with same alphabets are not different from each other at $P = 0.05$ analyzed by Tukey's HSD test. Error bars over the column represent the standard error ± SE of means. IC= Infected Control, HC= Healthy Control.

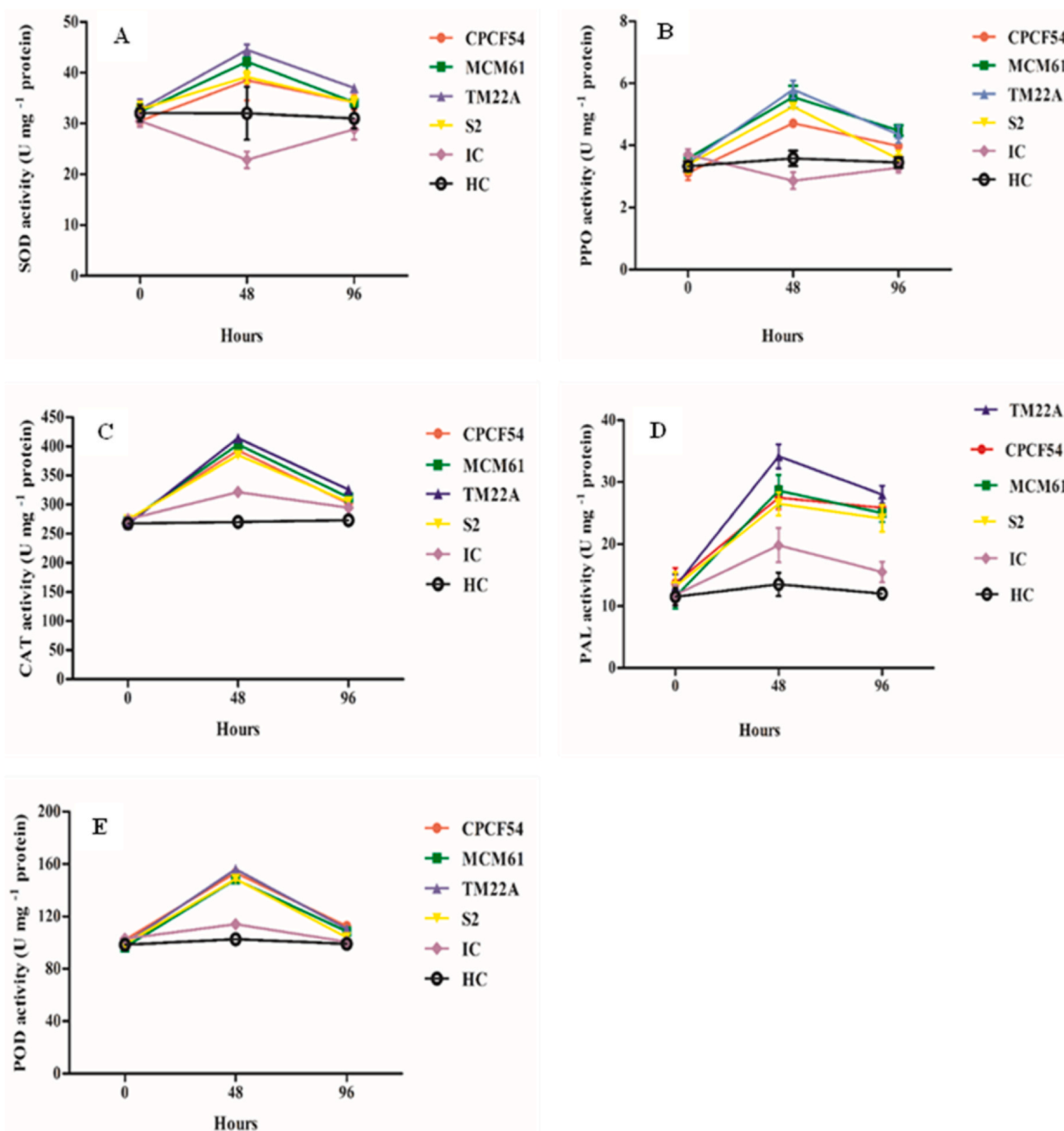

Fig. 6. Effect of lipopeptides (LPs) produced by *Bacillus* strains on defense enzyme activities of superoxide dismutase (SOD), polyphenol oxidase (PPO), catalase (CAT), phenylalanine ammonia lyase (PAL), and peroxidase (POD) in tomato fruit against *A. alternata*
Values are means of five replicates. Activities of SOD, PPO, CAT, PAL, and POD in tomato fruit were evaluated at 0, 48, and 96 h time intervals stored at 25 ± 2 °C. The error bars over the lines indicate the standard error ± SE of means.

33 % relative to healthy control treatment. The highest disease incidence 100 % was observed in infected control treatment. All tested *Bacillus* strains showed inhibition of *A. alternata* by suppressing the lesion diameter relative to infected control treatment. (Fig. 5B).

The results of *In Planta* assay correlate with *in vitro* plate assay where TM22A gave the highest inhibition of *A. alternata* relative to the control. Similar results were reported by (Arrebola, Sivakumar, & Korsten, 2010; Guardado-Valdivia et al., 2018; Jiang, Zhu, & Li, 2001), that support our work that LPs produced by *Bacillus* reduced the postharvest infection of avocado, citrus, and litchi fruit respectively. *B. velezensis* XT1 CECT 8661 reduced the disease development *B. cinerea* on grapefruit, strawberry, and tomato (Toral, Rodríguez, Béjar, & Sampedro, 2018). The antifungal effect of LPs have been reported in several previous reports (Farzand et al., 2019a,b; Fatima et al., 2023).

3.6. Enzyme activity assay

Tomato fruit subjected to treatment with *Bacillus* LPs were also tested for the activity of defense enzymes. All strains exhibited the same trend of POD, PAL, and CAT enzyme activity at 0, 48, and 96 h. The effect of *Bacillus* strains on the activity of antioxidant enzymes in tomato fruit revealed that the LPs enhanced the enzyme activity with the passage of time.

The SOD and PPO enzyme activity results revealed slightly different trends at different time intervals 0, 48, and 96 h. At the start of incubation, the enzyme activity of PPO and CAT increased steadily in all *Bacillus* treated fruit and reached a peak value at 48 h, TM22A strain showed higher enzyme activity relative to infected control. The activity of PPO and SOD in infected control treatment decreased gradually from 24 to 48 h and after 48 h the activity was increased again up to 96 h

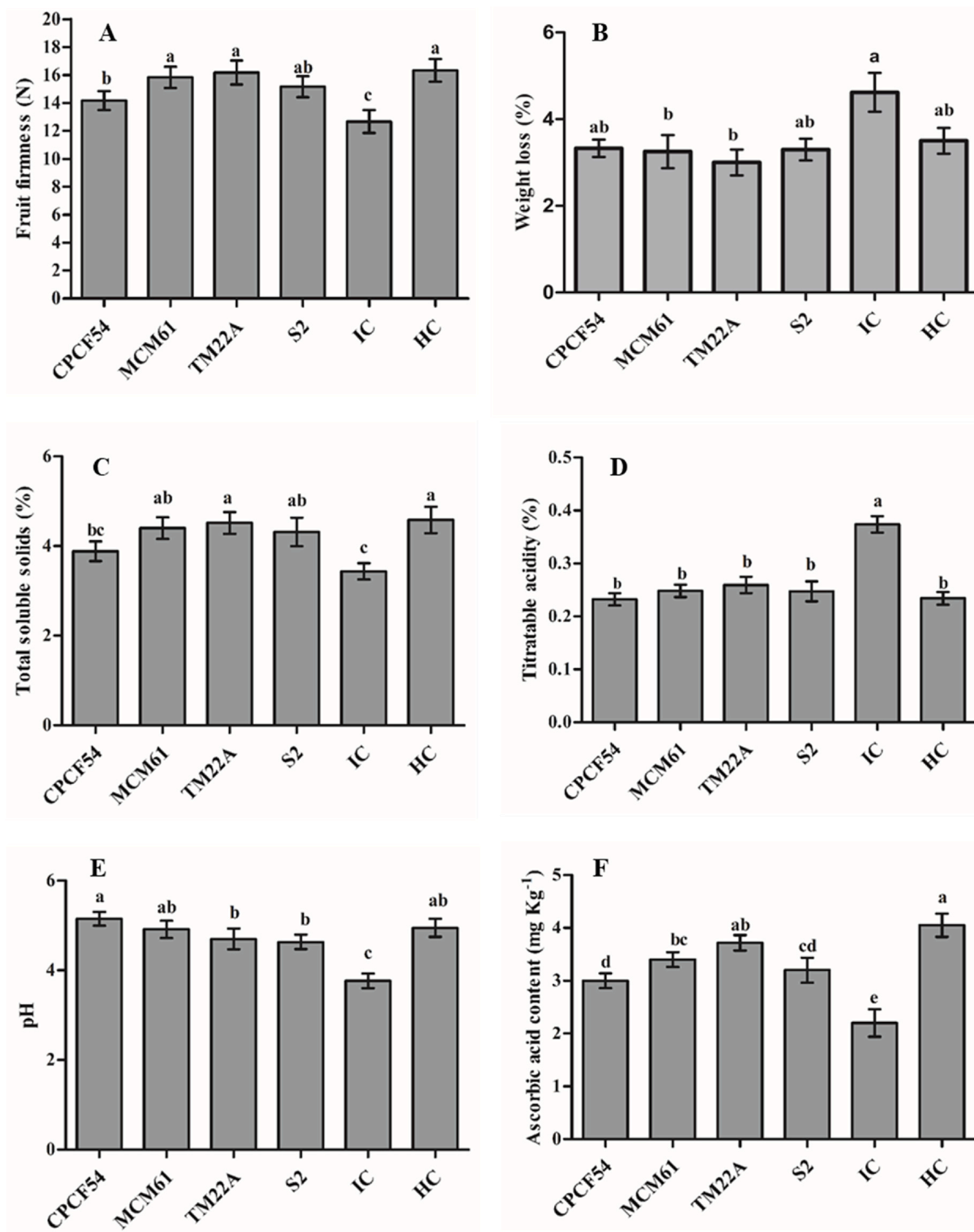

Fig. 7. Effects of lipopeptides (LPs) produced by *Bacillus* strains on postharvest quality tests of fruit firmness, weight loss, total soluble solids (TSS), titratable acidity (TA), pH, and ascorbic acid content in tomato fruit against *A. alternata*. Values are mean of six replicates and the alphabets over the column indicate that the means with same alphabets are not different from each other at $P = 0.05$ analyzed by Tukey's HSD test. Quality test in tomato fruit were evaluated at 0, 48, 96 h time intervals stored at 25 ± 2 °C. The error bars over the lines indicate the standard error ± SE of means. HC = Healthy Control, IC = Infected Control.

interval (Fig. 6A and B). The CAT and PAL activity also increased in *Bacillus* treated tomato fruit with a peak value at 48 h then it decreased gradually upto 96 h (Fig. 6C and D). At the early period of incubation, the activity of POD in the *Bacillus* treatment climbed steadily and reached a peak value at 48 h, TM22A strain showed the higher enzyme activity relative to healthy control treatment at 48 h. POD activity began to increase at 24 h and reached the highest at 48 h, but there was barely any change in the infected control treatment. After 48 h the enzyme activity was gradually declined (Fig. 6E).

To further unravel the potential of *Bacillus* LPs in defense elicitation the activities of CAT, SOD, PPO, POD, and PAL enzymes were evaluated in treated tomato fruit at 0, 48, and 96 h. *Bacillus* species promote plant defense against abiotic and biotic stresses by inducing the defense activity of antioxidants such as CAT, SOD, PPO, POD, and PAL (Rais,

Jabeen, Shair, Hafeez, & Hassan, 2017). According to a previous report, *Bacillus subtilis* M4 promotes defense response in tomatoes and cucumbers against *Pythium aphanidermatum* and *Colletotricum lagenarium* respectively (Ongena et al., 2005). In a similar study, Li, Hua, Liu, and Guo (2008), *B. subtilis* suppresses *Ralstonia solanacearum* in tomatoes by improving the activities of PPO, POD, PAL, SOD, and CAT enzymes.

The improvement of disease suppression can be achieved through the activation of the plant defense system (Cao, Zeng, & Jiang, 2006). Several poisonous phenols and flavonoids are produced by plants through the phenylpropanoid pathway, which is a significant metabolic process. POD enzyme takes part in the final stage of lignin formation and converts phenolic chemicals into poisonous quinones. PPO is another crucial enzyme that is involved in defense system and catalyzes the conversion of phenols and lignin into extremely toxic quinones that are poisonous to the pathogen (Moosa et al., 2019).

By dismutation of superoxide anion (O_2 radical dot), SOD can protect cells from oxidative stress and produce oxygen and H_2O_2. Additionally, during the defense processes, H_2O_2 may be involved in the oxidation of phenols (Chittoor, Leach, & White, 1999). CAT which turns hydrogen peroxide into O_2 and water, is a crucial part of oxygen scavenging systems (Heng-Moss et al., 2004). Earlier, Chandrasekaran, Belachew, Yoon, & Chun (2017), reported that the tomatoes treated with *Bacillus* BR05 effectively suppressed *X. campestris* pv, *vesicatoria* via induction of the activity of defense enzymes CAT, SOD, POD, and PPO enzymes in tomato. Therefore, it can be concluded that *Bacillus* derived LPs plays an important role in protecting the fruit from *A. alternata* infection during postharvest storage.

3.7. Postharvest quality analysis

The effect of LPs extracted from *Bacillus* strains was tested on fruit firmness, weight loss, total soluble solids, titratable acidity, ascorbic acid content, and pH of tomato fruit against *A. alternata*. All the tested *Bacillus* LPs altered fruit firmness and weight loss compared to control. The highest fruit firmness was showed by TM22A (16.1 N) and MCM61 (15.8 N) as compared to healthy control and the lowest fruit firmness was showed by infected control which was 12.6 N. The lowest weight loss was observed in TM22A (4.62 %) and S2 (4.50 %) treated fruit as compared to healthy control (4.74 %) and the highest weight loss was observed in infected control (3.79 %). Furthermore, the highest titratable acidity was observed in infected control treatment (0.37). The lowest values of titratable acidity were shown by TM22A (0.25 %) and MCM61 (0.24 %) relative to healthy control (0.23 %). On the other hand, the highest TSS was observed in TM22A (4.51 %) and MCM61 (4.4 %) treated fruit as compared to healthy control (4.58 %) and the TSS was the lowest in infected control treatment (3.43 %) (Fig. 7).

The pH value was the highest in CPCF54 (5.15), and MCM61 (4.91) treated fruit and the lowest (3.76) in infected control fruit relative to healthy control (4.95). The highest VIT C content was observed in TM22A (3.71 mg kg^{-1}) and MCM61 (3.4 mg kg^{-1}) relative to healthy control (4.05 mg kg^{-1}) and lowest VIT C content was observed in infected control fruit (2.2 mg kg^{-1}) (Fig. 7). Postharvest quality analysis of tomato fruit indicated that the LPs produced by *Bacillus* strains suppressed the lesion diameter and disease incidence of *A. alternata* with a very low impact on fruit quality fruit firmness, ascorbic acid, total soluble solids, weight loss, titratable acidity, and fruit pH relative to infected control treatment. *Bacillus* LPs have the potential to protect fruit quality and improve shelf life during storage (Zhang et al., 2022). Several previous studies have also reported such treatments which tend to increase fruit shelf life during storage and protect from disease with a very little impact on fruit quality (Moosa et al., 2022; Sukorini et al., 2013).

4. Conclusions

In conclusion, the LPs extracted from *Bacillus* strains possess the potential to suppress the development of Alternaria rot of tomato. The study presents a novel and promising *Bacillus altitudinis* strain TM22A with remarkable antifungal potential against Alternaria rot of tomato. The LPs extracted from *Bacillus* strains enhanced the activity of SOD, CAT, POD, PPO, and PAL in treated tomato fruit with little impact on fruit quality. The application of *Bacillus* derived antifungal LPs showed great potential as an alternative to synthetic chemicals for being health and environmentally safe to control Alternaria rot of tomato fruit. The outcome of this study is of great significance to guide the research and development of *Bacillus*-derived antifungal agents to suppress Alternaria rot of tomato. Future research should evaluate the molecular mechanisms associated with the use of LPs for controlling the postharvest Alternaria rot of tomato and other produce.

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CRediT authorship contribution statement

Javaria Malik: Performed experiments, Writing – original draft. **Anam Moosa:** Conceptualization, Methodology, Data curation, Writing – original draft, Supervision, Writing – review & editing. **Faisal Zulfiqar:** Conceptualization, Methodology, Data curation, Writing – original draft, Supervision, Writing – review & editing. **Muhammad Naveed Aslam:** Writing – review & editing. **Marzough Aziz Albalawi:** Writing – review & editing. **Sanaa Almowallad:** Writing – review & editing. **Tahir Mahmood:** Writing – review & editing. **Abdulrahman Alasmari:** Writing – review & editing. **Jean Wan Hong Yong:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

References

Ahmet, C., Saban, K., Hamdullah, K., & Ercan, K. (2005). Antifungal properties of essential oil and crude extracts of *Hypericum linarioides* Bosse. *Biochemical Systematics and Ecology, 33*, 245–256. https://www.sciencedirect.com/science/article/pii/S0305197804002522.

Alvarez, F., Castro, M., Príncipe, A., Borioli, G., Fischer, S., Mori, G., et al. (2012). The plant-associated *Bacillus* amyloliquefaciens strains MEP218 and ARP23 capable of producing the cyclic lipopeptides iturin or surfactin and fengycin are effective in biocontrol of sclerotinia stem rot disease. *Journal of Applied Microbiology, 112*(1), 159–174. https://doi.org/10.1111/j.1365-2672.2011.05182.x

Arrebola, E., Sivakumar, D., & Korsten, L. (2010). Effect of volatile compounds produced by *Bacillus* strains on postharvest decay in citrus. *Biological Control, 53*(1), 122–128. https://doi.org/10.1016/j.biocontrol.2009.11.010

Avdiushko, S. A., Ye, X. S., & Kuc, J. (1993). Detection of several enzymatic activities in leaf prints of cucumber plants. *Physiological and Molecular Plant Pathology, 42*(6), 441–454. https://doi.org/10.1006/pmpp.1993.1033

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Analytical Biochemistry, 72*(1–2), 248–254. https://www.academia.edu/download/52930778/livre.pdf#page=177.

Cao, J., Zeng, K., & Jiang, W. (2006). Enhancement of postharvest disease resistance in Ya Li pear (Pyrus bretschneideri) fruit by salicylic acid sprays on the trees during fruit growth. *European Journal of Plant Pathology, 114*, 363–370. https://doi.org/10.1007/s10658-005-5401-8

Catiempo, R. L., Photchanachai, S., Bayogan, E. R. V., & Vichitsoonthonkul, T. (2021). Possible role of nonenzymatic antioxidants in hydroprimed sunflower seeds under heat stress. *Crop Science, 61*(2), 1328–1339. https://doi.org/10.1002/csc2.20403

Chandrasekaran, M., Belachew, S. T., Yoon, E., & Chun, S. C. (2017). Expression of β-1, 3-glucanase (GLU) and phenylalanine ammonia-lyase (PAL) genes and their enzymes

in tomato plants induced after treatment with *Bacillus* subtilis CBR05 against Xanthomonas campestris pv. vesicatoria. *Journal of General Plant Pathology, 83*, 7–13. https://doi.org/10.1007/s10327-016-0692-5

Chitarra, G. S., Breeuwer, P., Nout, M. J. R., Van Aelst, A. C., Rombouts, F. M., & Abee, T. (2003). An antifungal compound produced by *Bacillus* subtilis YM 10–20 inhibits germination of Penicillium roqueforti conidiospores. *Journal of Applied Microbiology, 94*(2), 159–166. https://doi.org/10.1046/j.1365-2672.2003.01819.x

Chittoor, J. M., Leach, J. E., & White, F. F. (1999). Induction of peroxidase during defense against pathogens. *Pathogenesis-Related Proteins in Plants*, 171–193. https://www.academia.edu/download/52930778/livre.pdf#page=177.

Cozzolino, M. E., Distel, J. S., García, P. A., Mascotti, M. L., Ayub, M. J., Benazzi, L. M., et al. (2020). Control of postharvest fungal pathogens in pome fruits by lipopeptides from a *Bacillus* sp. isolate SL-6. *Scientia Horticulturae, 261*, Article 108957. https://doi.org/10.1016/j.scienta.2019.108957

Deleu, M., Paquot, M., & Nylander, T. (2005). Fengycin interaction with lipid monolayers at the air–aqueous interface—implications for the effect of fengycin on biological membranes. *Journal of Colloid and Interface Science, 283*(2), 358–365. https://doi.org/10.1016/j.jcis.2004.09.036

Farzand, A., Moosa, A., Zubair, M., Khan, A. R., Ayaz, M., Massawe, V. C., et al. (2020). Transcriptional profiling of diffusible lipopeptides and fungal virulence genes during *Bacillus* amyloliquefaciens EZ1509-mediated suppression of Sclerotinia sclerotiorum. *Phytopathology, 110*(2), 317–326. https://apsjournals.apsnet.org/page/authorinformation.

Farzand, A., Moosa, A., Zubair, M., Khan, A. R., Hanif, A., Tahir, H. A. S., et al. (2019a). Marker assisted detection and LC-MS analysis of antimicrobial compounds in different *Bacillus* strains and their antifungal effect on Sclerotinia sclerotiorum. *Biological Control, 133*, 91–102. https://doi.org/10.1016/j.biocontrol.2019.03.014

Farzand, A., Moosa, A., Zubair, M., Khan, A. R., Massawe, V. C., Tahir, H. A. S., et al. (2019b). Suppression of Sclerotinia sclerotiorum by the induction of systemic resistance and regulation of antioxidant pathways in tomato using fengycin produced by *Bacillus* amyloliquefaciens FZB42. *Biomolecules, 9*, 613. https://doi.org/10.3390/biom9100613

Fatima, R., Mahmood, T., Moosa, A., Aslam, M. N., Shakeel, M. T., Maqsood, A., et al. (2023). *Bacillus* thuringiensis CHGP12 uses a multifaceted approach for the suppression of Fusarium oxysporum f. sp. ciceris and to enhance the biomass of chickpea plants. *Pest Management Science, 79*(1), 336–348. https://doi.org/10.1002/ps.7203

Gao, Z., Zhang, B., Liu, H., Han, J., & Zhang, Y. (2017). Identification of endophytic *Bacillus* velezensis ZSY-1 strain and antifungal activity of its volatile compounds against Alternaria solani and Botrytis cinerea. *Biological Control, 105*, 27–39. https://doi.org/10.1016/j.biocontrol.2016.11.007

Goy, P. A., Felix, G., Metraux, J. P., & Meins, F., Jr. (1992). Resistance to disease in the hybrid Nicotiana glutinosa x Nicotiana debneyi is associated with high constitutive levels of β-1, 3-glucanase, chitinase, peroxidase and polyphenoloxidase. *Physiological and Molecular Plant Pathology, 41*(1), 11–21. https://doi.org/10.1016/0885-5765(92)90045

Guardado-Valdivia, L., Tovar-Pérez, E., Chacón-López, A., López-García, U., Gutiérrez-Martínez, P., Stoll, A., et al. (2018). Identification and characterization of a new *Bacillus* atrophaeus strain B5 as biocontrol agent of postharvest anthracnose disease in soursop (Annona muricata) and avocado (Persea americana). *Microbiological Research, 210*, 26–32. https://doi.org/10.1016/j.micres.2018.01.007

Guo, Q., Dong, W., Li, S., Lu, X., Wang, P., Zhang, X., et al. (2014). Fengycin produced by *Bacillus* subtilis NCD-2 plays a major role in biocontrol of cotton seedling damping-off disease. *Microbiological Research, 169*(7–8), 533–540. https://doi.org/10.1016/j.micres.2013.12.001

Gu, Q., Yang, Y., Yuan, Q., Shi, G., Wu, L., Lou, Z., et al. (2017). Bacillomycin D produced by *Bacillus* amyloliquefaciens is involved in the antagonistic interaction with the plant-pathogenic fungus Fusarium graminearum. *Applied and Environmental Microbiology, 83*(19), Article e01075-17. https://doi.org/10.1128/AEM.01075-17

Heng-Moss, T., Sarath, G., Baxendale, F., Novak, D., Bose, S., Ni, X., et al. (2004). Characterization of oxidative enzyme changes in buffalograsses challenged by Blissus occiduus. *Journal of Economic Entomology, 97*(3), 1086–1095. https://doi.org/10.1093/jee/97.3.1086

Jiang, C. H., Liao, M. J., Wang, H. K., Zheng, M. Z., Xu, J. J., & Guo, J. H. (2018). *Bacillus* velezensis, a potential and efficient biocontrol agent in control of pepper gray mold caused by Botrytis cinerea. *Biological Control, 126*, 147–157. https://doi.org/10.1016/j.biocontrol.2018.07.017

Jiang, Y. M., Zhu, X. R., & Li, Y. B. (2001). Postharvest control of litchi fruit rot by *Bacillus* subtilis. *LWT-Food Science & Technology, 34*(7), 430–436. https://doi.org/10.1006/fstl.2001.0758

Ju, J., Guo, Y., Cheng, Y., & Yaoc, W. (2022). Analysis of the synergistic antifungal mechanism of small molecular combinations of essential oils at the molecular level. *Industrial Crops and Products, 188*, Article 115612. https://doi.org/10.1016/j.indcrop.2022.115612

Ju, J., Xie, Y., Yu, H., Guo, Y., Cheng, Y., Chen, Y., et al. (2020). Synergistic properties of citral and eugenol for the inactivation of foodborne molds *in vitro* and on bread. *LWT, 122*, Article 109063. https://doi.org/10.1016/j.lwt.2020.109063

Kim, P. I., Ryu, J., Kim, Y. H., & Chi, Y. T. (2010). Production of biosurfactant lipopeptides Iturin A, fengycin and surfactin A from *Bacillus* subtilis CMB32 for control of Colletotrichum gloeosporioides. *Journal of Microbiology and Biotechnology, 20*(1), 138–145. https://doi.org/10.4014/jmb.0905.05007

Klein, B. P., & Perry, A. K. (1982). Ascorbic acid and vitamin A activity in selected vegetables from different geographical areas of the United States. *Journal of Food Science, 47*, 941–945. https://doi.org/10.1111/j.1365-2621.1982.tb12750.x

Li, S. M., Hua, G. G., Liu, H. X., & Guo, J. H. (2008). Analysis of defence enzymes induced by antagonistic bacterium *Bacillus* subtilis strain AR12 towards Ralstonia solanacearum in tomato. *Annals of Microbiology, 58*, 573–578. https://doi.org/10.1007/BF03175560

Lim, S. M., Yoon, M. Y., Choi, G. J., Choi, Y. H., Jang, K. S., Shin, T. S., et al. (2017). Diffusible and volatile antifungal compounds produced by an antagonistic *Bacillus* velezensis G341 against various phytopathogenic fungi. *Plant Pathology Journal, 33*(5), 488. https://doi.org/10.5423/PPJ.OA.04.2017.0073

Meena, K. K., Sorty, A. M., Bitla, U., Shinde, A. L., Kumar, S., Wakchaure, G. C., et al. (2023). Stress-responsive gene regulation conferring salinity tolerance in wheat inoculated with ACC deaminase producing facultative methylotrophic actinobacterium. *Frontiers in Plant Science, 14*, 10.3389%2Ffpls.2023.1249600.

Moosa, A., Sahi, S. T., Khan, S. A., & Malik, A. U. (2019). Salicylic acid and jasmonic acid can suppress green and blue moulds of citrus fruit and induce the activity of polyphenol oxidase and peroxidase. *Folia Hortic, 31*(1), 195–204. https://doi.org/10.2478/fhort-2019-0014

Moosa, A., Zulfiqar, F., & Siddique, K. H. (2022). Transcriptional and biochemical profiling of defense enzymes in Citrus sinensis during salicylic acid and cinnamon mediated suppression of green and blue mold. *Frontiers in Plant Science, 13*, Article 1048433. https://doi.org/10.3389/fpls.2022.1048433

Ongena, M., Duby, F., Jourdan, E., Beaudry, T., Jadin, V., Dommes, J., et al. (2005). *Bacillus* subtilis M4 decreases plant susceptibility towards fungal pathogens by increasing host resistance associated with differential gene expression. *Applied Microbiology and Biotechnology, 67*, 692–698. https://doi.org/10.1007/s00253-004-1741-0

Ongena, M., & Jacques, P. (2008). *Bacillus* lipopeptides: Versatile weapons for plant disease biocontrol. *Trends in Microbiology, 16*(3), 115–125. https://doi.org/10.1016/j.tim.2007.12.009

Ostry, V. (2008). Alternaria mycotoxins: An overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin Journal, 1*, 175–188. https://doi.org/10.3920/WMJ2008.x013

Petrasch, S., Silva, C. J., Mesquida-Pesci, S. D., Gallegos, K., Van Den Abeele, C., Papin, V., et al. (2019). Infection strategies deployed by Botrytis cinerea, Fusarium acuminatum, and Rhizopus stolonifer as a function of tomato fruit ripening stage. *Frontiers in Plant Science, 10*, 223. https://doi.org/10.3389/fpls.2019.00223

Rais, A., Jabeen, Z., Shair, F., Hafeez, F. Y., & Hassan, M. N. (2017). *Bacillus* spp., a bio-control agent enhances the activity of antioxidant defense enzymes in rice against Pyricularia oryzae. *PLoS One, 12*(11), Article e0187412. https://doi.org/10.1371/journal.pone.0187412

Romanazzi, G., Sanzani, S. M., Bi, Y., Tian, S., Martínez, P. G., & Alkan, N. (2016). Induced resistance to control postharvest decay of fruit and vegetables. *Postharvest Biology and Technology, 122*, 82–94. https://doi.org/10.1016/j.postharvbio.2016.08.003

Romero, D., De Vicente, A., Rakotoaly, R. H., Dufour, S. E., Veening, J. W., Arrebola, E., et al. (2007). The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus* subtilis toward Podosphaera fusca. *Molecular Plant-Microbe Interactions, 20*(4), 430–440. https://doi.org/10.1094/MPMI-20-4-0430

Sarwar, A., Brader, G., Corretto, E., Aleti, G., Abaidullah, M., Sessitsch, A., et al. (2018). Qualitative analysis of biosurfactants from *Bacillus* species exhibiting antifungal activity. *PLoS One, 13*(6), Article e0198107. https://doi.org/10.1371/journal.pone.0198107

Singh, J., Prasad, N., & Singh, S. K. (2017). Postharvest treatment of guava (Psidium guajava L.) fruits with boric acid and NAA for quality regulation during ambient Storage. *International Journal of Bio-Resource and Stress Management, 8*(2), 201–206. https://doi.org/10.23910/IJBSM/2017.8.2.1633a

Slathia, S., Sharma, Y. P., Hakla, H. R., Urfan, M., Yadav, N. S., & Pal, S. (2021). Post-harvest management of Alternaria induced rot in tomato fruits with essential oil of Zanthoxylum armatum DC. *Frontiers in Sustainable Food Systems, 5*, Article 679830. https://doi.org/10.3389/fsufs.2021.679830

Steinberg, G., Schuster, M., Gurr, S. J., Schrader, T. A., Schrader, M., Wood, M., et al. (2020). A lipophilic cation protects crops against fungal pathogens by multiple modes of action. *Nature Communications, 11*, 1608. https://doi.org/10.1038/s41467-020-14949-y

Sukorini, H., Sangchote, S., & Khewkhom, N. (2013). Control of postharvest green mold of citrus fruit with yeasts, medicinal plants, and their combination. *Postharvest Biology and Technology, 79*, 24–31. https://doi.org/10.1016/j.postharvbio.2013.01.001

Tang, Q., Bie, X., Lu, Z., Lv, F., Tao, Y., & Qu, X. (2014). Effects of fengycin from *Bacillus* subtilis fmbJ on apoptosis and necrosis in Rhizopus stolonifer. *Journal of Microbiology, 52*, 675–680. https://doi.org/10.1007/s12275-014-3605-3

Toral, L., Rodríguez, M., Béjar, V., & Sampedro, I. (2018). Antifungal activity of lipopeptides from *Bacillus* XT1 CECT 8661 against Botrytis cinerea. *Frontiers in Microbiology, 9*, 1315, 10.3389%2Ffmicb.2018.01315.

Um, S., Fraimout, A., Sapountzis, P., Oh, D. C., & Poulsen, M. (2013). The fungus-growing termite Macrotermes natalensis harbors bacillaene-producing *Bacillus* sp. that inhibit potentially antagonistic fungi. *Scientific Reports, 3*(1), 3250. https://doi.org/10.1038/srep03250

Vanama, S., Pesari, M., Rajendran, G., Gali, U. D., Rathod, S., Panuganti, R., et al. (2023). Correlation of the effect of native bioagents on soil properties and their influence on stem rot disease of rice. *Sustainability, 15*(15), Article 11768. https://doi.org/10.3390/su151511768

Wang, J., Gao, Q., Zhao, F., & Ju, J. (2023b). Repair mechanism and application of self-healing materials for food preservation. *Critical Reviews in Food Science and Nutrition*, 1–11. https://doi.org/10.1080/10408398.2023.2232877

Wang, J., Zhao, F., Huang, J., Li, Q., Yang, Q., & Ju, J. (2023). Application of essential oils as slow-release antimicrobial agents in food preservation: Preparation strategies, release mechanisms and application cases. *Critical Reviews in Food Science and Nutrition*, 1–26. https://doi.org/10.1080/10408398.2023.2167066

Wu, H., Zhao, F., Li, Q., Huang, J., & Ju, J. (2022). Antifungal mechanism of essential oil against foodborne fungi and its application in the preservation of baked food. *Critical Reviews in Food Science and Nutrition*, 1–13. https://doi.org/10.1080/10408398.2022.2124950

Yang, J., Jing, X., Wang, T., Di, J., Chen, L., & Wang, Y. (2020). Overactivation of glutamate consuming pathways in l-glutamate treated tomato fruits lead to resistance against Alternaria Alternata. *Postharvest Biology and Technology, 169*, Article 111311. https://doi.org/10.1016/j.postharvbio.2020.111311

You, Y., Zhou, Y., Duan, X., Mao, X., & Li, Y. (2022). Research progress on the application of different preservation methods for controlling fungi and toxins in fruit and vegetable. *Critical Reviews in Food Science and Nutrition*, 1–12. https://doi.org/10.1080/10408398.2022.2101982

Zhang, B., Xu, L., Ding, J., Wang, M., Ge, R., Zhao, H., et al. (2022). Natural antimicrobial lipopeptides secreted by *Bacillus* spp. and their application in food preservation, a critical review. *Trends in Food Science & Technology, 127*, 26–37. https://doi.org/10.1016/j.tifs.2022.06.009