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Management Fusarium Wilt Disease in Tomato by Combinations of *Bacillus amyloliquefaciens* and Peppermint Oil

Magdi A. A. Mousa [1,*], Kamal A. M. Abo-Elyousr [1,*], Ahmed M. K. Abdel Alal [2] and Nouf Owdah Alshareef [3]

[1] Department of Arid Land Agriculture, Faculty of Meteorology, Environment and Arid Land Agriculture, King Abdulaziz University, Jeddah 80208, Saudi Arabia
[2] Presidency of Graduate Studies and Scientific Research, King Abdulaziz University, Jeddah 80208, Saudi Arabia; amkali@kau.edu.sa
[3] Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah 80208, Saudi Arabia; noalshareef@kau.edu.sa
* Correspondence: mamousa@kau.edu.sa (M.A.A.M.); Ka@kau.edu.sa (K.A.M.A.-E.)

Abstract: The most important disease of tomato is Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL). To control this disease, this study examined the combined use of bacterial bioagents and peppermint oil (PO). Seven bacterial isolates were collected from tomato plant rhizospheres and tested in vitro against FOL. The highest growth inhibition against FOL was shown by isolate No.3. This isolate was identified using 16S rRNA sequencing gene as *Bacillus amyloliquefaciens* (BA). Peppermint oil tested at different concentrations (1, 2, and 3%) against FOL mycelial growth in vitro showed the highest inhibition at 3%. The effects of BA, PO, and BA + PO in vitro on the seed germination and seedling vigor index of the tomato cv. 'Tala F1' was also tested. All "BA, PO, and BA + PO" treatments increased the percentage of germinated seeds and seedlings' main shoots and root length compared to control treated seeds. The BA, PO, and BA + PO treatments were further tested under greenhouse and field conditions with pre-treated seedlings in FOL-contaminated soil. Under greenhouse conditions, each treatment decreased disease severity compared to untreated seedlings. Under field conditions, pre-treatment of tomato seedlings with BA and PO treatments reduced disease severity greater than BA + PO in combination and the mock-treated plants (66.6% for BA, 66.6% for PO and 55.3% for BA + PO, respectively). These findings support the use of BA or PO as bio-control agents against *F. oxysporum* in tomato. The interplay between peppermint oil, *B. amyloliquefaciens*, *F. oxysporum*, and the host plant requires further study to identify the causative mechanism for this increased disease resistance.

Keywords: tomato fusarium wilt; peppermint oil; *Fusarium oxysporum* f. sp. *lycopersici*; internal transcribed spacer; bacterial bioagent

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1. Introduction

Tomato (*Solanum lycopersicum* L.) is widely cultivated and is one of the most important vegetable crops, ranking next to potato in global vegetable cropping area and first amongst processing crops [1]. Tomato wilt disease, caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL), is the most harmful tomato disease leading to severe production losses [2]. Tomato Fusarium wilt is a soil-borne systemic disease severely affecting all plant growth stages and is the main limiting factor in both greenhouse and field-grown tomato production [3,4]. Disease incidence is 100% with complete infection of all plant tissues. The main symptoms observed are yellowing, wilting, stunting of growth, and ultimately death of plants [5]. *F. oxysporum* f. sp. *lycopersici*, the causative agent, includes three physiological races, which prove challenging to develop resistance to Fusarium wilt [6].

Fusarium pathogens have a wide variety of hosts and a large degree of morphological and physiological heterogeneity, enabling them to occupy different ecological niches in

diverse geographical regions [7]. Molecular approaches are now routinely used to identity *Fusarium* species, which are often more efficient than those based on morphological characteristics [8]. The use of internal transcribed spacer (ITS) region DNA sequence analysis has now become a standard routine for the discovery, identification, classification, and phylogenetic analysis of many fungi at the species level [9]. Molecular methods based on sequence analysis of multiple genes can be useful to classify and identify pathogenic isolate and identify new species [10].

An objective of modern farming is to produce quality food using sustainable and ecologically friendly approaches. This includes reducing the use of certain chemicals, such as pesticides, to minimize possible environmental harm. Biological agents and natural products, like essential oils, are an attractive choice for growth enhancers or as alternative pesticides for pest management [11]. The use of biological control agents is considered more sustainable, reduces harmful residues entering the food chain, safer for implementation and economical in cost, depending on the strength of protective antagonistic organisms against plant pathogen [12]. *Bacillus* spp. are widespread in soil and have been examined for their capacity as biocontrol agents (BCAs) against plant diseases [13]. Rhizobacteria play a significant role in plants, suppressing plant diseases and enhancing crop yield [14]. The identification of effective rhizobacteria isolates can be achieved with the use of 16S rRNA gene-based sequencing methods [15,16].

Essential oils produced from various plant species have also been suggested as potential biological control agents and shown to have antimicrobial activity and antioxidant and bio-regulatory properties [17]. Peppermint (*Mentha* × *piperita* or *Mentha balsamea* Wild.) is a medicinal plant with high nutritional value and use in both food and pharmaceutical industries [18]. The application of essential oils is considered an effective method for preventing Fusarium wilt in banana, muskmelon, and tomato [19,20]. Essential oils can be used as a protective biofungicide for *F. oxysporum* f. sp. *lycopersici* management, with the antifungal properties of the essential oils supporting disease resistance and promoting growth [21].

Our study aims to examine the antifungal activity of peppermint oil (PO) and *B. amyloliquefaciens* (BA) against FOL and analyze the chemical components of PO. We tested the use of PO, BA, and both (BA + PO) in combination to reduce the severity of tomato Fusarium wilt disease caused by FOL under greenhouse and field conditions, as well as to examine their effect on growth and yield parameters.

2. Materials and Methods

2.1. Source of Pathogenic FOL Isolate

Fusarium oxysporum f. sp. *lycopersici* isolate (FKAU1) used in all experiments in the presented study was isolated by [22] and has been shown to cause high disease severity of tomato wilt.

2.2. Molecular Characterization of F. oxysporum f. sp. lycopersici FKAU1

The pathogenic fungal isolate KFAU1 was purified by single spore isolation and identified based on its micro and macroscopic characteristics [23]. DNA was extracted and PCR was used to amplify the nuclear rDNA region spanning ITS1 and ITS2 in two rounds [24]. The first amplification was performed in a total reaction volume of 50 µL, including the following reagents: PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin); 200 µM dNTPs; 0.4 µM of primers ITS1 (3′-GTCAGTCCATTGGCTCTCTC-5′) and ITS2 (3′-TCCTTGACACCATCACAGAG-5′); 10 µL of template DNA solution; and one unit of Tag DNA polymerase (Toyobo, Japan). The PCR product from the second amplification was subjected to preparative electrophoresis in 1.5% agarose gel in TAE buffer. All amplifications yielded a single DNA product. The DNA product band was excised from the ethidium bromide-stained gel and purified using a JETSORB gel extraction kit (Gentaur Molecular Products BVBA, Kampenhout, Belgium). Direct sequencing of PCR products was conducted by an Applied Biosystems 373A sequencer and PRISM Dye Terminator Cycle Sequencing kit (Applied Biosystems, California, CA, USA).

The nucleotide sequence data of the ITS1 and ITS2 regions were subjected to pairwise alignment by the method of [25] using the program "GENETYX-MAC" (Genetyx Corp., Osaka, Japan) and sequences were further analyzed using BLAST from the National Center of Biotechnology Information (NCBI), National Library of Medicine (US), National Center for Biotechnology Information; Bethesda (MD), Available online: https://www.ncbi.nlm.nih.gov/, accessed on 26 October 2020).

2.3. Isolation and Purification of Rhizobacteria Isolates

Bacteria isolates were isolated from the soil rhizosphere of healthy tomato plants. Soil samples (10 g) were homogenized with 100 mL of sterile distilled water in a magnetic shaker for 30 min at 150 rpm then diluted into different concentrations (10^{-3}:10^{-9}) and well vortexed. The supernatant (1 mL) of two highest dilutions was poured on plates contained nutrient sucrose agar (NSA) medium (5.0 g L^{-1} peptone, 3.0 g L^{-1} beef extract, 5.0 g L^{-1} sucrose, 15 L^{-1} agar, pH adjusted to 7.0) and incubated at 28 ± 1 °C for 24–48 h. Visually distinct individual colonies were transferred to NSA medium to obtain single colonies and stored at 4 °C until use.

2.4. In Vitro Assessment of the Antifungal Activity of Rhizobacteria Isolates against FKAU1

Seven bacterial isolates were tested for their antifungal potential towards FKAU1 using the dual culture technique. Bacterial isolates were grown on NSA medium and incubated at 28 °C for 24 h. Pathogenic isolate KFAU1 was grown on PDA for 7 days at 25 °C. KFAU1 disks (5 mm diameter) were inoculated in the middle of Petri dishes (9 cm diameter) containing PDA medium. Individual bacterial cultures were transferred by inoculation loop in two lines on either side of the FKAU1 disk. Plates containing pathogen disks without bacterial inoculation served as control. All plates were incubated at 27 ± 1 °C. Each treatment replicated three times. Mycelial growth of FKAU1 was recorded. The following formula was used to measure the percent of growth inhibition [26]:

$$growth\ reduction\ (\%) = \frac{control\ growth - innoculated\ growth}{control\ growth} \times 100$$

2.5. Identification of Bio-Agent Isolate Using 16S rRNA Sequencing Gen

The bio-agent isolate showing the highest FOL growth reduction was sent to Solgent Company, Daejeon South Korea for rRNA gene sequencing. DNA was extracted and isolated using SolGent bead purification. The ribosomal rRNA gene was amplified by PCR with two universal primers for bacterial amplification: forward primer 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492r (5′-TACGGYTACCTT GTTACGACTT-3′). PCR products were purified using a PCR purification kit (Cosmo Genetech, Republic of Korea) and reconfirmed by electrophoreses on 1% agarose gel. Bands were excised and sequenced in both sense and antisense directions using 27f and 1492r primers [27]. Sequences were analyzed using BLAST from the National Center of Biotechnology Information (NCBI).

2.6. In Vitro Antifungal Activity of Peppermint Oil against Fusarium oxysporum f. sp. lycopersici FKAU1

The antifungal potential of peppermint oil (PO) towards FOL FKAU1 was tested at three concentrations (1, 2, and 3%). PO, obtained from local market Jeddah, KSA, was dissolved in acetone (1:10 mL) and blended to create a stock solution. Stock PO in acetone was applied to conical flasks containing 100 mL of sterilized PDA medium before solidification to obtain required concentrations before being poured into Petri dishes (9 cm diameter). FKAU1 discs (5 mm diameter) were placed in the center of Petri dishes and incubated at 25 ± 1 °C. Plates containing PDA medium with acetone without PO served as the control. Each treatment contained three replicates. The reduction of mycelial growth was determined in comparison to the growth in control [11].

2.7. Effects of B. amyloliquefaciens (BA) and Peppermint Oil (PO) on Seed Germination and Seedling Vigor In Vitro under FKAU1 Infection

The in vitro effect of seed pre-treatment with BA (isolate No. 3, shown to be highly antagonistic potential towards KFAU1), PO (3%, concentration showing the highest FKAU1 growth reduction), and BA in combination with PO (BA + PO) was examined in four treatments. Five groups of seeds of the tomato cv. 'Tala F1′ were separately treated as follows: (1) group 1 seeds soaked in a BA suspension ($1 \times l0^8$ cfu mL^{-1}), (2) group 2 seeds dipped in an aqueous solution of 3% PO, (3) group 3 seeds treated with a combination of BA and PO, (4) group 4 seeds soaked in with FKAU1 as a negative control, and (5) group 5 seeds soaked in sterile distilled water (served as a control). For all treatments, seeds were gently shaken for 30 min. Each treatment was replicated 3 times with 50 seeds per replicate. Following treatment, seeds were placed on wet filter paper and incubated at 28 °C for one week. The percentage of germinated seeds, mean shoot length (MSR), and mean root length (MRL) were recorded according [28]. The seedling vigor index (VI) was determined using the equation as follows:

$$VI = (MSL + MRL) \times germination\%$$

2.8. Chemical Analysis of Peppermint Oil

Gas chromatography mass spectrometry (GC-MS) was used to evaluate peppermint oil at the Analytical Chemistry Unit, Faculty of Science, Assiut University, Egypt. GC (Clarus 600—Perkin Elmer, Palo Alto, CA, USA), MS ((Model 6890 N/5975 B—Agilent Technologies, Palo Alto, CA, USA) were attached to a DB-S 30 m × 0.25 mm × 0.25 µm) GC column (Perkin Elmer, USA). GC parameters were as follows: column temperature raised from 40 °C to 220 °C at a rate of 4 °C min^{-1}, 250 °C injector temperature, 1 µL injection volume, and 280 °C transfer temperature. MS parameters were as follows: EI mode, 70 eV ionization voltage, 180 °C ion source temperature, and 50–600 Da scan range. Peaks were recognized using NIST and Wiley Registry 8 Edition, based on library search. Both mass spectral and retention data of the known standards obtained under similar GC-MS conditions confirmed the identity of certain components (Wiley GC-MS 275 libraries). Internal standards were implemented allowing an estimation of the yield for the selected compound based on the standard calibration curve.

2.9. Effect of B. amyloliquefaciens (BA) and Peppermint Oil (PO) on Disease Severity Percentage of Tomato Fusarium Wilt under Greenhouse Conditions

2.9.1. Preparation of Pathogen Inoculum FKAU1

Sterilized 0.5 L high-density polyethylene bottles containing barley grain medium (100 g of barley kernels, 80 mL of water, and autoclaved at 121 °C for 20 min on two subsequent days) were inoculated with FKAU1 and incubated at 25 °C for 15 days. Pots (20 cm diameter) were sterilized and filled with 1.5 kg of sterile mixture of soil: sand (1:1 w/w) and fertilizer 1% NPK (12:4:6) and combined with FKAU1 barley grain medium (2% w/w) seven days before seedling transplantation as per [22].

2.9.2. Preparation of *B. amyloliquefaciens* (BA) for Seedling Pre-Treatment

BA isolate for seedling pre-treatment was grown in 200 mL flasks containing 100 mL of nutrient sucrose broth (5.0 g L^{-1} peptone, 3.0 g L^{-1} beef extract, 5.0 g L^{-1} sucrose) broth incubated at 28° C with agitation for 48 h. Bacterial cultures were centrifuged at 10,000 rpm for 10 min and resuspended in water to a density of $1 \times l0^8$ cfu mL^{-1}.

2.9.3. Pots Experiment

Greenhouse experiments were performed at Hada Al-Sham Agricultural research station, King Abulaziz University, Jamoum Governorate, Kingdom of Saudi Arabia (lat. 21.796, long. 39.722) from 16 October 2018 to 25 March 2019 and from 20 October 2019 to 1 April 2020. Seeds were sown in 20 cm diameter plastic pots containing a mixture of soil: sand (1:1 w/w) and 1% NPK (12:4:6). First, 28-day-old seedlings of the tomato variety

'Tala F1' were soaked for 10 min in either BA solution (1×10^8 cfu mL^{-1}), 3% PO solution, BA + PO solution, or water (control treatment) before transferred into pots containing prepared FKAU1. Each treatment was replicated 3 times, 3 pots per replicate, and 1 seedling per pot. All pots were placed on a bench in a climate-controlled greenhouse at 30 + 5 °C with 68–80% RH and watered as required. After 60 days from planting, disease severity was scored using a modified numerical scale proposed by Sallam et al. (2019) as follows: (0) no symptoms, (1) ≥25% of leaflets yellowed and dark brown root bundles, (2) 25–50%, (3) 50–75%, and (4) 75–100%. Disease severity and severity reduction (%) were calculated by the following formulae [22]:

$$Disease\ severity\ percentage = \frac{\sum[n \times V]}{C \times N} \times 100$$

where n is the number of plants within each infection type, V is numerical values of infection category, N is the total number of plants examined, and C = highest numerical category (4):

$$Disease\ reduction\ percentage = \frac{c - t}{c} \times 100$$

where c is the mean disease severity of untreated plants and t is the disease severity of treated plants.

2.9.4. Field Experiments

Field experiments were performed at Hada Al-Sham Agricultural research station, King Abulaziz University, Jamoum Governorate, Kingdom of Saudi Arabia (lat. 21.796, long. 39.722) from 16 October 2018 to 25 March 2019 and from 20 October 2019 to 1 April 2020. A randomized block design was followed with field plots (3 m × 3 m) consisting of 2 rows and 4 plants per row (8 plants/plot), three plots per treatment. Tomato seedlings (Tala F1) were treated as in the greenhouse experiment previously described before transplantation to field positions. Disease severity was recorded 60 days from planting. At harvest, the cumulative yield for all plants per treatment and fresh and dry weight of all plants were recorded.

2.10. Statistical Analysis

Analysis of variance was conducted using the Statistical Analysis System (SAS) program (ver. 9.00, SAS Institute, Cary, NC, USA). The treatment means were compared by F-test and the Least Significant Differences test (LSD) at the 5% probability level [29].

3. Results

3.1. Identification of Isolate KFAU1 of the Pathogen

FOL isolate FKAU1 was molecularly characterized using ITS sequencing (ITS1 and ITS2). BLAST search on the NCBI data libraries for similarities to the ITS sequences showed the highest matches to *Fusarium oxysporum* isolate NIHHS467 (GenBank accession No. KY555030.1) and *F oxysporum* strain ATCC 48112 (GenBank accession No. KU729043.1) with 100% sequence similarity. The ITS sequence of isolate FKAU1 has been lodged within the GenBank sequences database under accession no. MW165780.1 (Table 1).

Table 1. BLAST molecular characterization of pathogenic isolate FKAU1 by ITS analysis.

Bioagent Isolate	Maximum Score	Total Score	Query Cover	E Value	Percent Identity	Most Similar Organism	GenBank Accession No.
FKAU1	974	974	100%	0.0	100%	*Fusarium oxysporum*	KY555030.1

3.2. Effect of Antagonistic Bacteria on Radial Growth of Fusarium oxysporum FKAU1 In Vitro

The seven bacterial isolates from tomato plant rhizosphere tested for their antagonistic effect against FKAU1 in vitro indicate that each was able to inhibit the growth of FKAU1

compared to the control at different degrees (7–45%) (Figure 1). Bacterial isolate No. 3 showed the highest inhibition percentage of FKAU1 growth, around 45%. Bacterial isolates No. 1, 5, and 6 showed equally moderate inhibition of growth. Isolate No. 3 was selected for further characterization and chosen as a potential bio-agent against FKAU1.

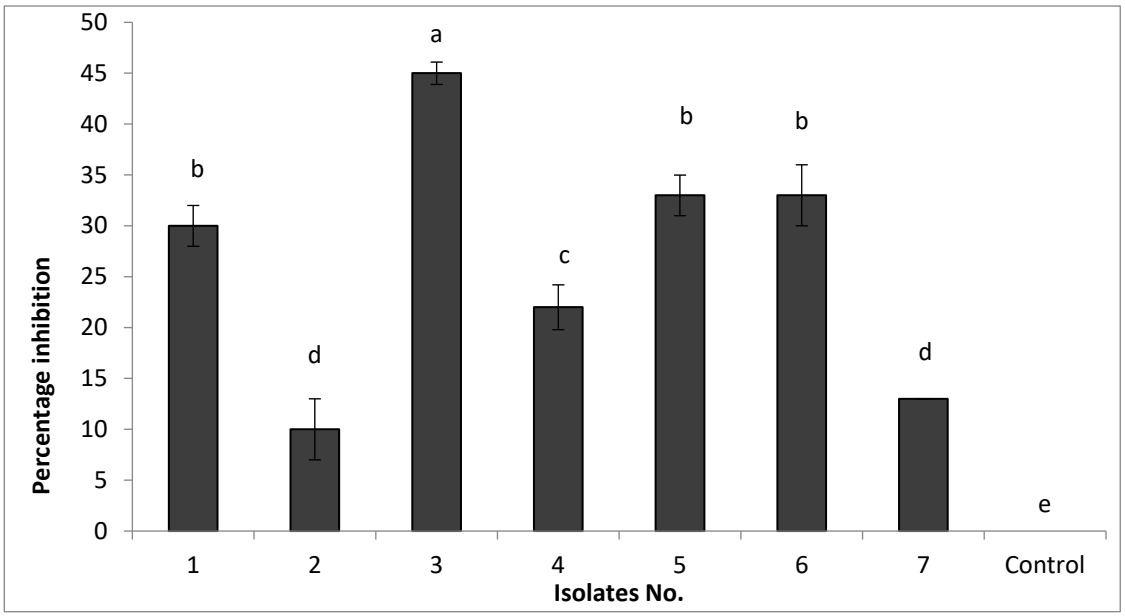

Figure 1. Percentage of *Fusarium oxysporum* growth inhibition caused by different bacterial strains in vitro. Bars indicate the standard error, three replicate plates were used for each treatment and the experiment was repeated twice. Columns with the same letters are not significantly different according to Fisher's protected least significant difference at $p \leq 0.05$.

3.3. Identification of Antagonistic Isolate Using 16S rRNA

Bioagent isolate No. 3 was molecularly characterized using 16S rRNA sequencing. BLAST search on the NCBI data libraries (16S ribosomal RNA sequences (Bacteria and Archaea) and non-redundant nucleotide collection) for similarities of the 16S rRNA sequences showed it to be most similar to *Bacillus amyloliquefaciens* strain MPA1034 (GenBank accession No. KT758463.1) with 100% percent identity query coverage (Table 2).

Table 2. Molecular characterization of unknown isolates by 16S rRNA analysis.

Bioagent Isolate	Maximum Score	Total Score	Query Cover	E Value	Percent Identity	Most Similar Organism	GenBank Accession No.
KAUBL2	1552	1552	100%	0.0	100%	*Bacillus amyloliquefaciens*	MH283880.1

3.4. Antifungal Activity of Peppermint Oil (PO) against Fusarium oxysporum FKAU1 In Vitro

The growth of FKAU1 was suppressed at all tested concentrations (1, 2, and 3%) compared with untreated controls (Figure 2), with 3% showing the greatest reduction at 35%.

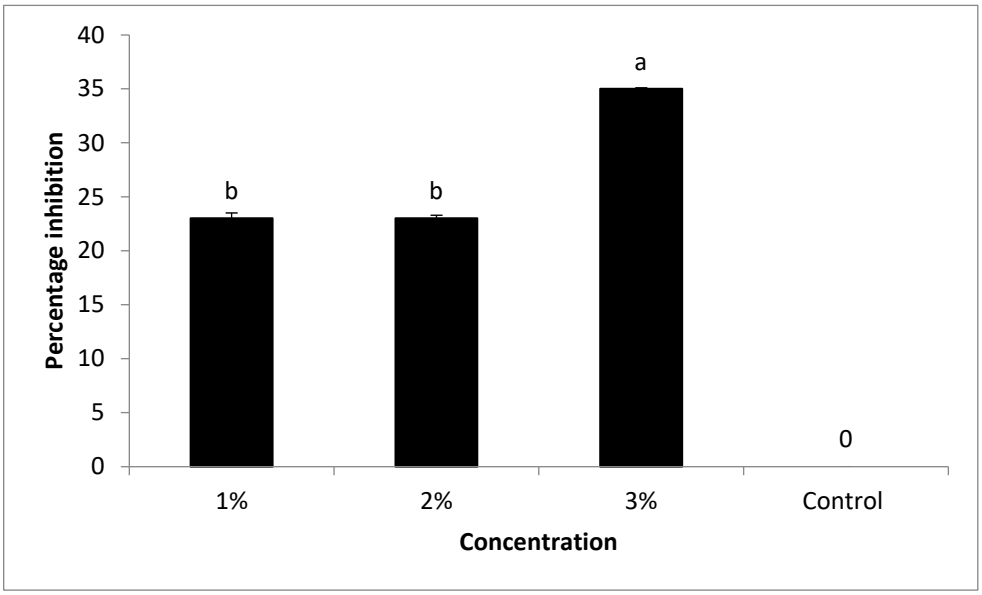

Figure 2. Percentage of *Fusarium oxysporum* growth inhibition caused by different concentrations of peppermint oil in vitro. Bars indicate the standard error, three replicate plates were used for each treatment and the experiment was repeated twice. Columns with the same letters are not significantly different according to the least significant difference at $p \leq 0.05$.

3.5. GC-MS Analysis of Peppermint Oil

A total of 17 compounds were able to be identified (Table 3). The main components sorted by the estimated yield were cyclohexanol, 5-methyl-2-(1-methylethyl)-(28.47%), cyclohexanone, 5-methyl-2-(1-methylethyl)-(19.91%), 1-menthone (14.53%), and 4-cyanoben zophenone (6.95%). All other constituents were below the 6% yield.

Table 3. Qualitative and quantitative profile of peppermint oil.

Compound	RT	Yield (%)
α-Pinene	6.394	2.03
D-Limonene	8.883	5.51
Cyclohexanone, 5-methyl-2-(1-methylethyl)-	12.437	19.91
1-Menthone	12.746	14.53
Cyclohexanol, 5-methyl-2-(1-methylethyl)-	12.958	28.47
Aceptophenone	14.050	2.98
Cyclohexene, 4-methyl-1-(1-methylethyl)-	16.305	6.03
Benzene, 1, 2, 3-trimethyl-	16.889	2.48
1H-Indole, 5-methyl-2-phenyl-	39.994	1.84
4-Cyanobenzophenone	40.097	6.95
Benzamide, N-[6-(2-furyl)-2-oxo-2H-pyran-3-yl]-	40.200	2.51
Acetic acid, [4-(1, 1-dimethyl) phenoxy]-, methyl ester	40.389	2.16
Silane, triethyl (2-phenylethoxy)-	41.047	1.18
Tert-Butyl (5-isopropyl-2-methylphenoxy) dimethylsilane	41.298	1.35
Acetic acid, oxo ((1-phenylethyl)amino)-, hydrazide	41.356	0.60
1,4 Bis (trimethylsilyl) benzene	41.453	1.16
Acetic acid, [4-(1, 1-dimethylethyl) phenoxy]-, methyl ester	41.476	0.31

RT = retention time.

3.6. Effect of BA Bacillus amyloliquefaciens and Peppermint Oil on In Vitro on Seed Germination and Seedling Vigor

The BA treatment had the greatest effect, improving both germination (+27%) and seedling vigor, followed by PO treatment, with a smaller increase for the BA + PO treatment (Table 4) over the mock-treatment control. MSL and MRL were also increased by both BA

and PO with a similar trend, with BA + PO showing the least improvement in MSL and no increase in MRL.

Table 4. Effect of *Bacillus amyloliquefaciens* (BA), peppermint oil (PO) and BA + PO in combination on seed germination and seedling vigor under Fusarium wilt infection.

Treatments	Germination%	MSL (cm)	MRL (cm)	Vigor Index (VI%)
Ba	75 a (+27)	1.9 (±0.4) [a]	5.5 (±0.9) [a]	555
Po	70 b (+22%)	1.5 (±0.2) [b]	4.2 (±0.7) [b]	399
BA + Po	65 c (+17%)	1.3 (±0.1) [c]	3.2 (±0.5) [c]	292.5
Infected control	88 a (+83%)	1.6(±0.2) [b]	4.1(±0.7) [b]	501.6
Healthy control	48 d	1.2 (±0.1) [d]	3.1 (±0.4) [c]	206.4

Mean shoot length (MSL), mean root length (MRL), vigor index (VI). Means (±SE) followed by different lettering indicates differences ($p \leq 0.05$) between treatments determined by ANOVA LSD.

3.7. Effect of Bacillus amyloliquefaciens and Peppermint Oil on Disease Reduction of Tomato Fusarium Wilt Severity under Greenhouse Conditions

Pre-treatment of seedlings with BA, PO, and BA + PO before planting in soil infected with FKAU1 significantly decreased the wilt disease severity percentage compared to untreated seedlings (Table 5). BA treatment reduced Fusarium wilt disease intensity (52.9%). PO and BA + PO equally reduced disease severity to a lesser degree than BA alone (41.1 and 38.8%, respectively).

Table 5. Inhibition of Fusarium wilt disease by seedling pre-treatment with *Bacillus amyloliquefaciens* (BA), 3% peppermint oil (PO), or in combination (BA + PO) under greenhouse conditions.

Treatments	Severity (%)	Reduction%
BA	40 (±1.2) [c]	52.9
PO	50 (±2.0) [b]	41.1
BA + PO	52 (±1.9) [b]	38.8
Infected control	85 (±2.4) [a]	-

Means (±SE) followed by different lettering indicates differences ($p \leq 0.05$) between treatments determined by ANOVA LSD.

3.8. Effect of Bacillus amyloliquefaciens and Peppermint Oil on Disease Severity under Field Conditions

Evaluation of BA, PO, and BA + PO combinations on wilt disease severity under field conditions indicated that pre-treatment of tomato seedlings with each treatment resulted in a reduction of Fusarium wilt disease severity when compared to untreated controls (Table 6).

Table 6. Inhibition of Fusarium wilt disease by seedling pre-treatment with *Bacillus amyloliquefaciens* (BA), 3% peppermint oil (PO), or in combination (BA + PO) under field conditions.

Treatment	Severity (%)	Reduction (%)
BA	5(±1.1) [b]	66.6
PO	5 (±1.4) [b]	66.6
BA + PO	7(±1.5) [b]	53.3
Untreated	15 (±1.9) [a]	-

Means (±SE) followed by different lettering indicates differences ($p \leq 0.05$) between treatments determined by ANOVA LSD.

3.9. Effect of Bacillus amyloliquefaciens and Peppermint Oil on Tomato Fruit Yield under Field Conditions

Tomato plants treated with BA resulted in a higher yield compared to other treatments (40.74 ton/ha) while treatment with PO alone was similar to untreated plants.

Treatment with BA + PO in combination increased both the yield and fruit number; however, the yield was not high as the BA treatment alone (Table 7).

Table 7. Fruits/plant and total fruit yield (ton/ha) of the tomato cv. 'Tala F1' as affected by Fusarium wilt disease and seedlings pre-treatment with *Bacillus amyloliquefaciens* (BA), 3% peppermint oil (PO), or in combination (BA + PO) under fusarium infestation field conditions.

Treatment	Yield (ton/ha)	Fruit/Plants
BA	40.74 (±6.1) [a]	33.4 (±2.3) [a]
PO	33.36 (±4.4) [c]	29.3 (±2.1) [b]
BA + PO	35.13 (±5.3) [b]	32.1 (±2.7) [a]
Untreated	34.76 (±4.5) [c]	29.9 (±2.0) [b]

Means (±SE) followed by different letters significantly differ according to the LSD test ($p \leq 0.05$).

4. Discussion

In our study, we identified the highly pathogenic isolate FKAU1 previously isolated by [22] using ITS sequencing, based on the amplification of the rDNA region. FKAU1 isolate showed a 100% sequence similarity with *F. oxysporum* NIHHS467 and *F. oxysporum* strain ATCC 48112.

To develop potential bio-control agents against FKAU1, we obtained seven bacterial isolates from the rhizosphere of healthy tomato plants. Rhizosphere microbial populations are known to play essential roles in plant health and disease protection [30] and are a source of potential bio-control agents. The capability of these seven isolates to inhibit the growth of FKAU1 was tested by the dual culture technique [31,32]. Our results agree with those reported by Kang et al. [33], who mentioned that *Bacillus* spp. have been considered potential biocontrol agents for the control of phytopathogens because they have various antifungal activities and are safe to use. Our results indicate that all tested isolates were able to inhibit the growth of FOL to differing degrees. A range of mechanisms are potentially behind this antagonistic activity and the inhibition of FOL, such as a greater potential for nutrient competition or by the release of products in the media, resulting in a reduced growth rate or killing *F. oxysporum* [34], or the production of antibiotics that can suppress the growth of pathogens [34].

Bacterial isolate No. 3 showed the highest percentage of inhibition of FKAU1 growth in vitro and was selected for further study. To identify the species of this isolate, we sequenced the 16S rRNA region, which suggested close relation to *Bacillus amyloliquefaciens* [16]. Our results agree with those reported previously by [8,22].

Different concentrations (1, 2, and 3%) of peppermint oil were tested in vitro against the growth of FKAU1. The findings in the current study are based on that by increasing oil concentration, the antifungal activity of the oil increased. Under laboratory screening, the oil has previously been shown to be effective against *F. oxysporum* [35]. These results are agree with [36], who reported that peppermint oil has shown potent inhibitory activity against several microorganisms, such as *Penicillium digitatum*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *A. flavus*, *Mucor* spp., *Candida albicans*, and *Fusarium oxysporum*. The chemical analysis of peppermint oil was conducted using GC-MS. Antimicrobial activity is attributed to some of the main components and the resulting synergistic or antagonistic action. However, minor components may also contribute to the biological activity. The antimicrobial activity of essential oil is perhaps attributable to α-Pinene, γ-Terpinene, Estragole, Methsuximide, Undecane, (3, 4-Dimethoxyphenyl) (3H-imidazol-4-yl) Methanone, Caryophyllene, Eugenol, Phenol, Azulene, 4, 8-dimethyl-6-phenyl-, 1-eicosene, D-limonene, Aceptophenone, 1H-Indole, 5-methyl-2-phenyl-, acetic acid, [4-(1, 1-dimethyl) phenoxy], methyl ester, and Silane, triethyl (2-phenylethoxy). These compounds have known antimicrobial properties [37]. Some of the other detected components of investigated essential oils are known bactericides [38] and may contribute to antimicrobial activity.

Volatile oil compounds have antimicrobial effects by either directly harming pathogen cells or by disrupting cellular metabolism [39]. It was reported that by responding to the active sites of cellular enzymes, volatile compounds may act as H+-carriers, leading to adenosine triphosphate depletion and disturbing cellular membranes [40,41]. Due to these antimicrobial activities, volatile oil compounds may also hurt beneficial bacteria.

The antifungal activity of BA, PO, or both in combination were tested on tomato seed germination and seedling vigor in vitro. Pre-treatment of seeds before infection with FKAU1 displayed increased seed germination, MSL, and MRL when compared to the mock treatment. BA gave the highest seed germination and seedling vigor followed by PO. Treatment of seeds with *Bacillus* spp cultures has previously been shown to lead to increased shoot and root dry weight of tomato plants compared to untreated and infected seeds with *Fusarium oxysporum* [42]. It has been proposed that the production of antibiotics by *Bacillus* spp. plays an essential part in the suppression of plant diseases since these strains can synthesize a wide range of antifungal metabolites. Among them are cyclic lipopeptides (CLPs) that include members of the fengycin surfactin, and iturin families [34]. The results herein agree with previous research [12,30]

In our greenhouse experiment, we determined that BA, PO, and BA + PO seedling treatments were all effective in reducing the severity of Fusarium wilt compared with untreated plants (Table 5). Treating seedlings with BA or PO separately resulted in a greater reduction in disease severity than in plants treated with BA and PO combined. The most effective treatment showing the lowest Fusarium wilt disease severity was BA. The results of Ajilogba et al. [42] showed that tomato plant treated with *B. amyloliquefaciens* reduced tomato Fusarium wilt by 75% compared to the infected control. Previously, *Bacillus* spp. has shown significant reductions in the incidence or severity of various diseases on a diversity of hosts via some mechanisms [43]. Roots treated with *Bacillus* isolates had more root hair compared to those untreated [42]. Suppression of disease by *Bacillus* spp. may be based on the production of a wide array of secondary metabolites (antibiotics, nonvolatile and volatile compounds) and show great potential by colonizing the root surface, causing fungal mycelia lysis, and promoting plant growth [44]. Major components of essential oils, such as menthone and menthol, may also be responsible for inhibiting the growth of the fungal pathogen. In addition, as has already been proposed, other minor components may work together synergistically in oil [36,45].

The treatments trialed in the greenhouse experiment were further tested under field conditions. Similar to previous experiments, all treatments showed BA seedling pre-treatment was best at reducing disease severity, followed by PO, and again the combination of BA + PO ranking third.

Our results are similar to [46], who reported that *B. amyloliquefaciens* SN16-1, isolated from soil, can prevent tomato wilt disease. This isolate is an important biocontrol agent as it can reduce the Fusarium wilt disease rate by 44.44% [47]. Rhizobacteria biocontrol agents are capable of solubilizing phosphate, aiding with plant nutrition, and generating phytohormone IAA, inducing systemic acquired resistance in plants and as such show promise as biocontrol agents [47,48]. *Bacillus* isolates that control plant disease and increase crop yield are highly desirable to improve the sustainability of agro-ecosystems [40].

5. Conclusions

Bacillus amyloliquefaciens and 3% peppermint oil reduced *F. oxysporum* f. sp. *lycopersici* (FKAU1) radial growth in vitro and when used as seedling pre-treatment for tomato cv. 'Tala F1' promoted seed germination and seeding vigor. Moreover, both treatments separately and in combination enhanced the growth and yield of tomato under greenhouse field conditions. These results are promising for the use of *B. amyloliquefaciens* and 3% peppermint oil as bio-control agents against *F. oxysporum* KFAU1. The interplay between peppermint oil, *B. amyloliquefaciens*, KFAU1, and the host plant requires further study to identify the causative mechanism of this increased disease resistance.

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