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Antagonistic Activity of *Bacillus atrophaeus* (MZ741525) against Some Phytopathogenic Microorganisms

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FIFTEEN bacterial isolates were isolated from soil samples collected from Matrouh Governorate, Egypt and screened for antagonistic activity against some bacterial and fungal plant pathogens. Isolate SM3 showed the highest inhibitory effect against the tested plant pathogens. This isolate was identified using 16S rRNA encoding gene sequence analysis as *Bacillus atrophaeus* (GenBank accession no.: MZ741525). Its antagonistic activity was confirmed against four soft rotting bacterial isolates namely: *Erwinia carotovora* subsp. *carotovora* (*Ecc*) isolates: Pep7C, Cab21B2 and Cab45B and *Erwinia chrysanthemi* (*Echr*) isolate Car1B as well as four fungal plant pathogens namely: *Curvularia lunata*, *Fusarium oxysporum*, *Aspergillus awamori* and *Penicillium* sp. The antagonistic effect of *B. atrophaeus* SM3 on the fungal pathogens was also investigated using the mycelial reduction test where the highest mycelial reduction percentage was recorded against *C. lunata* (93.4%) followed by *Penicillium* sp. (77.8%) then *F. oxysporum* (61.7%) and *A. awamori* (41.0%).Transmission Electron Microscopy examination of the fungal isolates antagonized by *B. atrophaeus* SM3 showed different morphological changes including cell shrinking, alterations in the cell wall thickness, light-scattering cytoplasm, and cytoplasmic reduction. Analysis of the crude extract of *B. atrophaeus* SM3 by GC-mass spectroscopy indicated the presence of many compounds such as Tridecanoic acid (TDA), Pyrrole, Pentadecanoic acid and different types of fatty acids, with known biological activity for controlling pathogenic microorganisms. These results indicate that *B. atrophaeus* SM3 could be used as an eco-friendly biocontrol agent for successful plant disease management strategies.

Keywords: 16S rRNA, *Bacillus atrophaeus*, Biological control, GC-mass, Phytopathogens, Soil bacteria.

Introduction

Plants are exposed to multiple pathogenic microorganisms on a global scale. Plant pathogenic bacteria have a deleterious effect on plants and induce substantial annual losses in crop production (Sundin et al., 2016). For example, bacterial soft rot disease caused by *Ec* subspecies (renamed *Pectobacterium carotovorum*) and *Echr* (renamed *Dickeya* spp.) affects a wide variety of crops (Youdkes et al., 2020), including potatoes and other economically important food crops. In addition to soft rot disease, these bacteria also cause blackleg of potato, foot rot of rice, and bleeding canker of pear (Charkowski, 2018).

Fungal diseases are also a significant global threat to various crop yields. More than 150 economically significant crop species are affected by the severe vascular wilt disease caused by *F. oxysporum* , of which the most significant susceptible crops are cotton, bananas, tomatoes, melons, and watermelons (Bertoldo et al., 2015). *C. lunata* also causes various plant diseases including leaf spots of corn in USA (Garcia-Aroca et al., 2018), early blight of tomato in Egypt (AbdElfatah et al., 2021), and leaf blight of mulberry in Thailand (Bussaban et al., 2017).

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Another fungal pathogen, *A. awamori* has been reported to cause guava fruit rot (Akhtar et al., 2018). Additionally, postharvest fruit rot was found to occur by many *Penicillium* species with the most economically impacted fruits being citrus (Bhatta, 2021), grapes (Ghuffar et al., 2021), apple and pear (Louw & Korsten, 2014).

Plant diseases must to be controlled to maintain the level of crop productivity (Toppo & Naik, 2015). Biological control of plant pathogenic microorganisms is becoming a critical need due to increased worldwide trade (Daranas et al., 2019), along with climate changes and insufficient protection of plant products leads to major crop losses. Thus to reduce the widespread use of chemical pesticides for controlling crop disease, prioritizing the identification of eco-friendly alternatives and plant protection methods that are hygienically safe for foods (Ongena & Jacques, 2008). Since many pesticide chemicals persist and accumulate in natural ecosystems, which represent a threat to animal and human health (Glick, 1995). Furthermore, the development of pesticide resistant fungal strains has been attributed to the misuse of fungicides (Kumar et al., 2012). Therefore, it is crucial to explore certain antagonists found in harsh and vulnerable climate zones that could protect crops and withstand biotic and abiotic stresses (Agarwal et al., 2017). Antibiosis of different bacterial species such as *Rahnella aquatilis* (El-Hendawy et al., 2005), *Pseudomonas fluorescens* (El-Hendawy et al., 1998; El-Hendawy & Abo-Elyousr, 2016), *Agrobacterium radiobacter, Bacillus subtilis* and *B.cereus* (Toppo & Naik, 2015), against a variety of plant pathogenic microorganisms have been reported. Different species of the genus *Bacillus* are classified as harmless and they have the potential to synthesize several compounds that have been effectively used for crop protection and industrial purposes (Shafi et al., 2017). The antagonistic activity of *Bacillus* spp. is attributed to different mechanisms, including the production of bioactive compounds, to prevent the growth of phytopathogenic fungi and bacteria (Ntushelo et al., 2019). Volatile metabolites which stimulate induced systemic resistance in plants, have been reported as produced by *Bacillus* spp. (Compant et al., 2005). Members of the genus *Bacillus* also antagonize fungal pathogens by several mechanisms such as competing for vital nutrients and generating fungitoxic products (Ntushelo et al., 2019). Additionally, secondary metabolites secreted by *Bacillus* spp. have antimicrobial, anticancer, and antifungal (Mondol et al., 2013).

This study, an antagonistic bacterial isolate, was isolated from a soil sample collected from Matrouh Governorate, Egypt, and its potential use as a biological agent for controlling some phytopathogenic bacteria and fungi was investigated.

Materials and Methods

Plant pathogenic bacteria and fungi used in this study

Four soft rotting bacterial isolates and four plant pathogenic fungal isolates were obtained from the culture collection of both the bacteriology and mycology laboratories of the Botany and Microbiology Department, Faculty of Science, Helwan University, Egypt and used in the present study. The soft rotting bacteria are *Erwinia carotovora* subsp. *carotovora* isolates: Pep7C, Cab21B2 and Cab45B were isolated from pepper and cabbage, respectively, whereas *Erwinia chrysanthemi* isolate Car1B was isolated from a rotted carrot root. The fungal isolates are, *Curvularia lunata* and *Aspergillus awamori*, isolated from soil, *Fusarium oxysporum*, isolated from potato and *Penicillium* sp. isolated from lemon. The host and references of these plant pathogens are presented in Table 1.

Soil samples collection and bacterial isolation

Soil samples were collected from different localities in Matrouh Governorate, Egypt. After removing approximately 50mm of the surface layer, the samples were collected in sterile plastic bags, transferred to the laboratory, and air-dried. Then bacteria were isolated from the soil according to Mew et al. (1976) as follows: Ten grams of the soil were added to 40ml of sterilized distilled water in a 250mL flask, the suspension was shaken for 10min and then was allowed to stand for 1min; 10mL of the soil supernatant were added to 90mL sterile distilled water in a 250mL flask and 10-fold serial dilutions in sterile distilled water were prepared. A volume of 100µL of each dilution was spread on the surface of nutrient agar plates. The plates were incubated at 30^{o}C±2 for 1-2 days; all the developed colonies were isolated, symbolized as SM1, up to SM15, purified, and maintained on nutrient agar slopes, at 4^{o}C.

TABLE 1. Plant pathogenic bacteria and fungi used in the present study

Species	Isolates	Host	References
Pathogenic bacteria			
Erwinia carotovora subsp. *Carotovora*	Pep7C	Pepper Fruits	(El-Hendawy et al., 2002)
Erwinia carotovora subsp. *Carotovora*	Cab21B2	Cabbage Leaves	(El-Hendawy et al., 2006)
Erwinia carotovora subsp. *Carotovora*	Cab45B	Cabbage Leaves	(El-Hendawy et al. , 2006)
Erwinia chrysanthemi	Car1B	Carrot roots	(El-Hendawy et al., 2002)
Pathogenic fungi			
Curvularia lunata	Cur	Soil	(Elsaba et al., 2022)
Fusarium oxysporum	Fus	Potato	Unpublished data
Aspergillus awamori	Asp	Soil	Unpublished data
Penicillium sp.	Pen	Lemon	Unpublished data

Screening of the soil bacteria for antagonistic activity against the soft rotting bacteria

Fifteen soil bacterial isolates were screened for their antagonistic activity against the plant pathogenic bacteria listed in Table 1. according to Rao et al. (2017). Briefly, 0.5mL of 10^8 CFU/mL of bacterial pathogen suspension, prepared from an overnight shaken culture, was spread on the surface of nutrient agar plates and the antibiosis of the soil bacterial isolates was tested by the agar well diffusion method, a well of 0.8mm was made on the nutrient agar plate surface by using a sterile cork borer, and each well received 50µl of a 24h old bacterial suspension containing 1.5×10^8 CFU/mL. Plates were then incubated at 37°C for 24h, and the size of the inhibition zones that developed around each well were measured. Each experiment was repeated three times. The bacterial isolate SM3 showed the highest inhibitory effect against the challenged bacterial isolates, therefore was used for the rest of this study.

Screening of the bacterial isolate SM3 for antagonistic activity against phytopathogenic fungi

The antagonistic effect of the bacterial isolate SM3 was tested against four pathogenic fungal isolates namely, *C. lunata*, *F. oxysporum*, *A. awamori* and *Penicillium* sp. using a dual culture plate assay (Bhattacharya et al., 2016). A 6mm diameter fungal disc of 7day old culture, of each fungus, was inoculated on the center of potato dextrose agar (PDA) plates, and a loopful of 24h old SM3 bacterial isolate was inoculated on four sides around the fungal disc on the PDA plate then incubated at 30°C±2 for 3-5 days. Antagonism was determined by observing the inhibition of fungal growth. Plates with pathogenic fungi alone were served as controls.

Mycelial growth inhibition test by isolate SM3 in liquid medium

A Mycelial growth inhibition test was performed for four fungal phytopathogens separately by inoculating 20mL of potato dextrose broth media using a 6mm diameter fungal disc excised from a 7 days old fungal colony. Then, 1mL (10^8 CFU) of an overnight suspension of the bacterial isolate SM3 was inoculated into the vials and incubated for 5 days at 28°C±2, control vials did not contain the bacterial suspension. After incubation, the fungal mycelium was harvested and dried to a constant weight in an oven at 60°C. The antagonistic effect of the bacterial isolate SM3 was determined by measuring the percentage of fungal mass reduction compared with the control according to the following formula: % reduction = Dry weight of control fungus - Dry weight of treated fungus) / Dry weight of control fungus ×100 (Adebola & Amadi, 2010).

Transmission Electron Microscopy (TEM) examination of phytopathogenic fungi treated with isolate SM3

A JEOL 1010 Transmission Electron Microscope (USA) at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt, was used to examine the antagonistic effect of the bacterial isolate SM3 against the four tested fungal phytopathogens. The specimens were prepared for TEM according to Bozzola & Russell (1992).

Molecular identification of the antagonistic bacterial isolate SM3

DNA extraction and polymerase chain reaction (PCR)

Since isolate SM3 showed the highest antagonistic activity against the bacterial and the fungal phytopathogens tested in this study, it was subjected to identification by 16S rRNA encoding gene sequence analysis. Briefly, DNA was extracted from 1.5mL of an overnight broth culture according Prabha et al. (2013). The quality and quantity of the extracted DNA were assessed using 1% agarose gel electrophoresis in 1× TAE buffer. PCR was performed in a final reaction volume of 50µl using Cosmo red master mix (Willowfort:WF-1020201) according to the manufacturer's instructions. Amplification of DNA fragment corresponding to the 16S rRNA encoding gene was performed by using 1µL of the extracted DNA as a template. Both 27F and 1492R, which span the full length of the 16S rRNA gene were used (Lagier et al., 2015). The amplification reactions were performed in Thermal Cycler (Biometra, Germany) for 35 cycles of denaturation (95°C), annealing (50°C), extension (72°C) each for 20 sec. The cycles were preceded by initial denaturation (95°C) for 3 min and terminated by an extended extension cycle at 72°C for 10min. The PCR product was electrophoresed, and a single fragment of ~1500 bp was amplified and then cut off from the gel and purified using PCR-M clean up system (VIOGENE cat# PF1001, Taiwan) according to the manufacturer's protocol. The purified DNA fragment was sequenced by providing the 27F-16S rRNA primer for strand sequence initiation using Sanger technology at GATC Company. Bacterial identification was verified through the National Center for Biotechnology Information (NCBI) nucleotide Basic Local Alignment Search Tool. (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Sequencing data analysis

The obtained nucleotide sequences were aligned to the total nucleotide collection of NCBI using the Basic Local Alignment Search Tool for nucleotide blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree is constructed using the UPGMA tree build method with 100 bootstrapping in Geneious 11.1.5 software (Kearse et al., 2012).

Identification of secondary metabolites of isolate SM3 by gas chromatography-mass spectrometry (GC–MS)

Preparation of crude extract of isolate SM3

Five ml of an overnight bacterial suspension of the bacterial isolate SM3 was inoculated into 250ml nutrient broth at the concentration of 10^8CFU/mL and incubated for 24h by shaking at 140rpm and 30°C±2°C. Then, the bacterial cells in the suspension were sonicated using a sonicator (Biologics, Inc., USA), then centrifuged for 20min at 10,000rpm and 4°C. Then, the supernatant was transferred to another vial for metabolite extraction. Extraction was performed using ethyl acetate at a ratio of 1:1 v/v for 24h, and the solvent was evaporated under pressure at 60°C using an RV 10 rotary evaporator (IKA, Germany) to obtain the crude extract containing the secondary metabolites. Finally, the crude extract was transferred to a clean vial, covered with foil, placed in a desiccator at room temperature to dry, and then maintained at 4°C until further analysis (Makuwa & Serepa-Dlamini, 2021).

GC-MS analysis

The GC-MS analysis was carried out using gas chromatography-mass spectrometry instrument stands with the following specifications, Instrument: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a thermo mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system was equipped with a TR-5 MS column (30m x 0.32mm i.d., 0.25µm film thickness). Analyses were carried out using helium as carrier gas at a flow rate of 1.0mL/min and a split ratio of 1:10 using the following temperature program: 60°C for 1 min; rising at 4.0°C/min to 240°C and held for 1min. The injector and detector were held at 210°C. Diluted samples (1:10 hexane, v/v) of 1µl of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 40-45, identification of the chemical constituents of the essential oil was deconvoluted using AMDIS software (www.amdis.net) and identified by its retention indices (relative to n-alkanes C8-C22), mass spectrum matching to authentic standards (when available), Wiley spectral library collection and the National Institute of Standards and Technology Research Library).

Results

Screening of the soil bacteria for antagonistic activity against the soft rotting bacteria

Fifteen bacterial isolates were isolated from soil samples collected from Matrouh Governorate, Egypt, and screened for antagonistic activities toward four soft rotting bacterial isolates. The soil bacterial isolate SM3 showed the most pronounced antimicrobial activity. Its antagonistic activity against *Ecc* isolates: Pep7C, Cab21B2 and Cab45B and *Echr* isolate Car1B was confirmed by formation of clear zone of 2, 7, 2 and 3mm, respectively. Cab21B2 was the most susceptible soft rotting isolate (Fig.1).

Screening of the bacterial isolate SM3 for antagonistic activity against the fungal isolates

The antagonistic activity of the bacterial soil isolate SM3 was determined against four fungal phytopathogens using a dual culture plate assay. Isolate SM3 restricted the growth of all the tested fungi, namely, *C. lunata, F. oxysporum, A. awamori and Penicillium* sp. (Fig. 2).

Mycelial growth inhibition test by isolate SM3 in liquid medium

The antagonistic effect of the bacterial isolate SM3 against the fungal pathogens was also investigated using mycelial growth inhibition test in potato dextrose broth. The mycelial growth of the four treated fungi was reduced compared with the control. The highest percentage of mycelial growth reduction was recorded against *C. lunata* (93.4%) followed by *Penicillium* sp. (77.8%), then *F. oxysporum* (61.7%) and *A. awamori* (41.0%) (Table 2).

TEM examination of phytopathogenic fungi treated with isolate SM3

TEM examination revealed the distortion of all four the fungal isolates treated with the antagonistic bacterium SM3 compared with the untreated control. The distortion appeared as vacant mycelia with distortion of the cell wall, loss of cell constituents (Figs. 3-6).

Molecular identification of antagonistic bacterial isolate SM3

The amplified fragments of the 16S subunit of the rRNA encoding gene of soil isolate SM3 was ~1500 bp (Fig. 7A**)**. The blast alignment and the constructed phylogenetic tree for the nucleotide sequences showed 100% identity and query coverage homology to *B. atrophaeus* MT125710 and MW740434 strains at 100% bootstrapping value, therefore, the bacterium was identified as *B. atrophaeus* SM3. The sequence was submitted to the NCBI GenBank and acquired an accession number; MZ741525 (https://www.ncbi.nlm.nih.gov/nuccore/MZ741525, accessed on 10 August 2021) (Fig. 7B**)**.

GC-MS analysis

The crude extract of *B. atrophaeus* SM3 strain was analyzed by gas chromatography mass spectrometry to determine the chemical constituents that could be involved in the antimicrobial activity of *B. atrophaeus* SM3. The results showed the presence of 17 bioactive compounds with minor component 3,7,11-Trimethyl-1-dodecanol and major compound of Pyrrolo[1,2-a]pyrazine-1,4-dione hexahydro-3-(2-methylpropyl) (Table 3 and Fig.8).

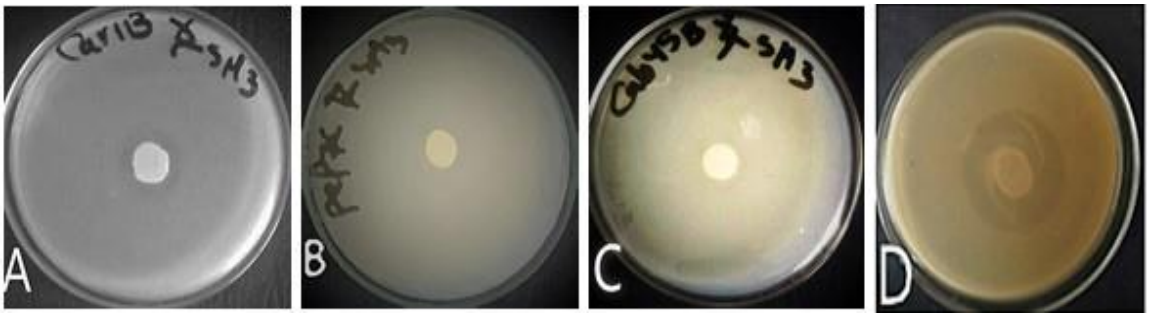

Fig. 1. Antibacterial activity of the bacterial isolate SM3 against four plant pathogenic bacteria. A) *Erwinia chrysanthemi* Car1B (B, C and D) *Erwinia carotovora* subsp. *carotovora* pep7C, Cab45B and Cab21B2, respectively, on NA plate, incubated at 30°C for 24h

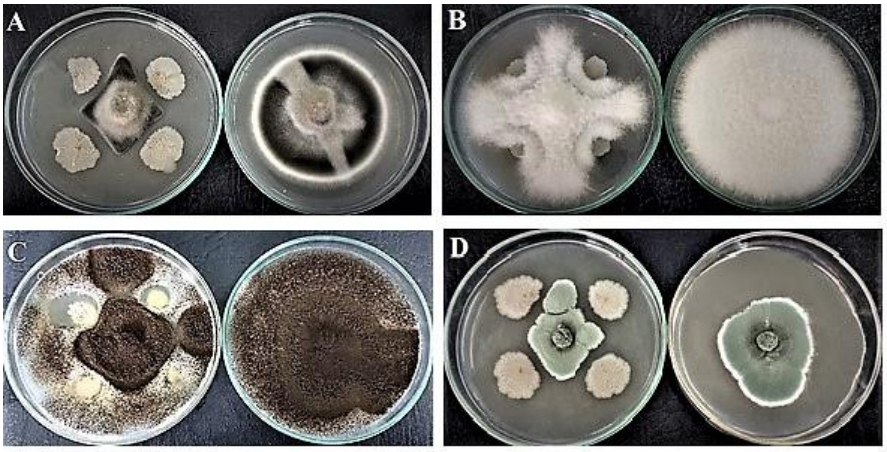

Fig. 2. In vitro evaluation of the antagonistic activity of the bacterial isolate SM3 against four plant pathogenic fungi compared with the control. A) *Curvularia lunata*, B) *Fusarium oxysporum*, C) *Aspergillus awamori* and D) *Penicillium* sp.

TABLE 2. Mycelial growth inhibition test by isolate SM3 in liquid medium

Fungal strain	Control dry weight (g)	Treated dry weight (g)	% of reduction
Curvularia lunata	0.50±0.10	0.033±0.06	93.4
Fusarium oxysporium	0.12±0.05	0.046±0.05	61.7
Aspergillus awamori	0.10±0.04	0.059±0.10	41.0
Penicillium sp.	0.09±0.04	0.020±0.04	77.8

*Dry weight is presented as means of three independent replica (g) ± SD

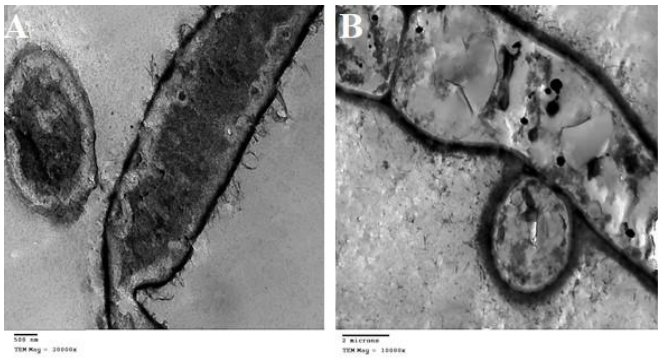

Fig. 3. TEM examination of *Curvularia lunata*. A) control (20000 X), B) treated (10000 X)

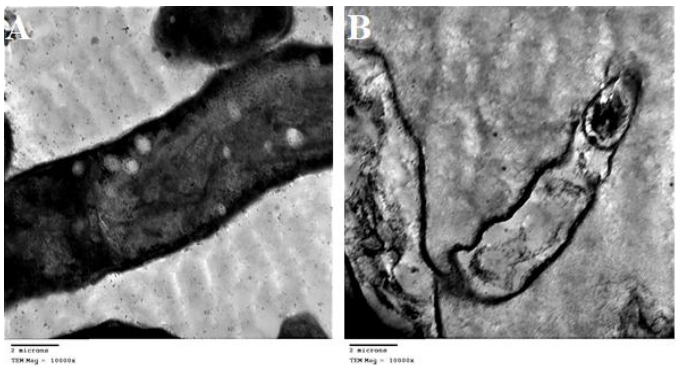

Fig. 4. TEM examination of *Aspergillus awamori*. A) control (10000 X), B) treated (20000 X)

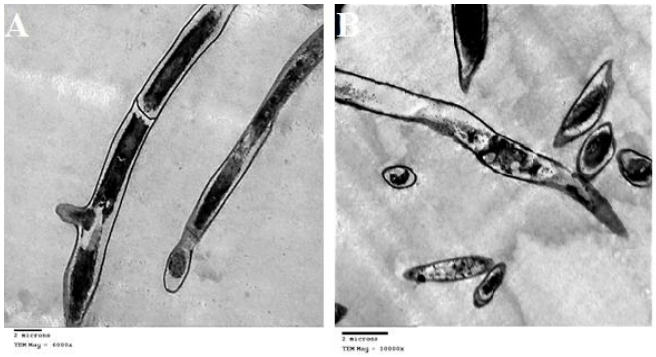

Fig. 5. TEM examination of *Fusarium oxysporum.* A) control (600 X), B) treated (10000 X)

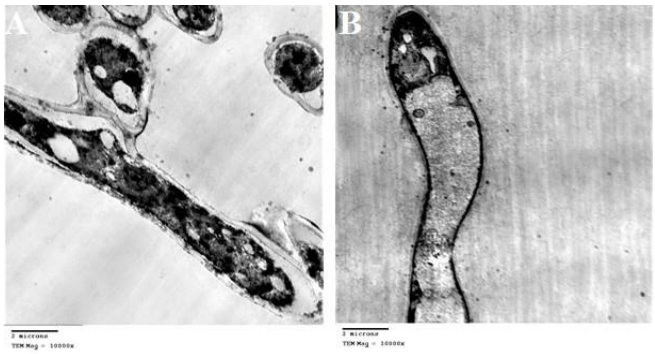

Fig. 6. TEM examination of *Penicillium* sp. A) control (10000 X), B) treated (10000 X)

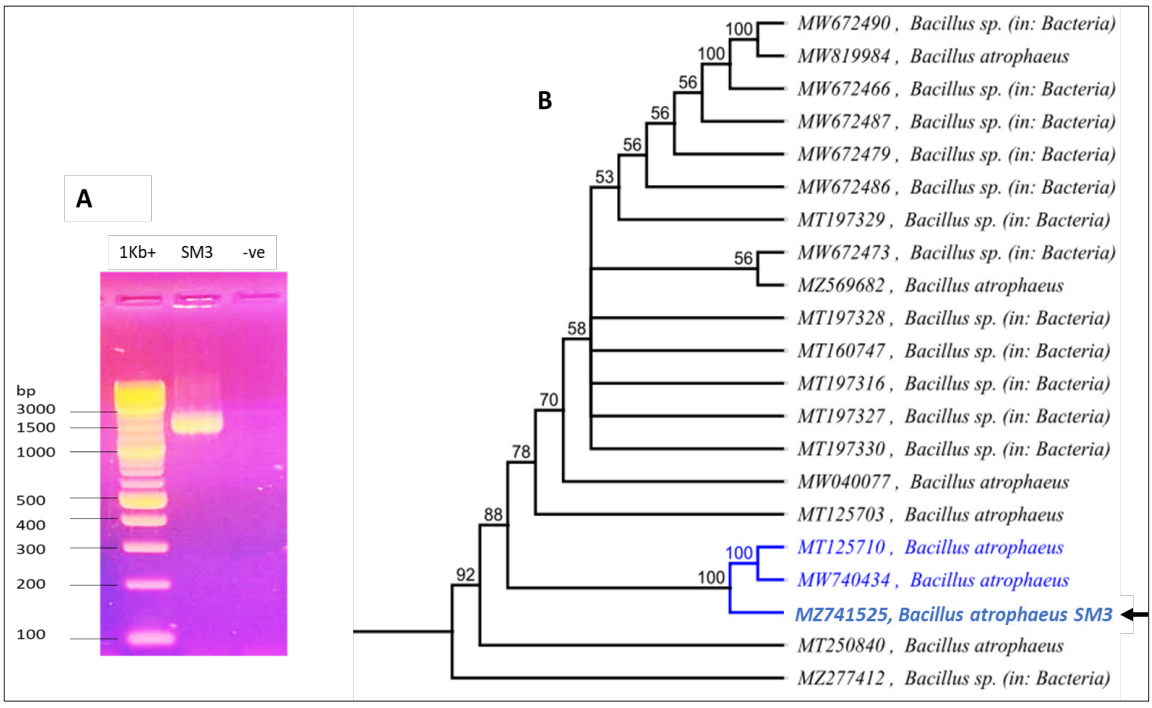

Fig. 7. Molecular identification of SM3 sample. (a) gel electrophoresis showing PCR amplification products from 16S rRNA encoding genes. SM3 refers to the amplified product, -ve refers to the negative control, and no amplifications confirm no PCR contamination. 1kb+ refers to DNA ladder (*BioLabs*). (b) Phylogenetic tree of the 16S rRNA encoding genes. The phylogeny indicates that the sequence belongs to *Bacillus atrophaeus* labelled in blue font color (arrow)

TABLE 3. GC–MS analysis of *B. atrophaeus* crude extract

Name	Formula	Molecular weight	Retention time	Area %	Nature of compounds
(S)-4-Methylhexanoic acid-	$C_7H_{14}O_2$	130	4.14	2.61	Branched fatty acids (Murai et al., 1985)
Chloroxylenol 4-Chloro-3,5-dimethylphenol	C_8H_9OCl	156	9.88	1.33	Phenols (Chippaux et al., 1996)
Nizatidine	$C_{12}H_{21}N_5O_2S_2$	331	11.96	2.71	Histamine H2 receptor antagonists (Gatta et al., 2019)
Tridecanoic acid	$C_{13}H_{26}O_2$	214	14.45	1.00	A fatty acid methyl ester and a dodecanoate ester (Chowdhury et al., 2021)
Amino-ethyl thiosulphuric acid	$C_2H_7NO_3S_2$	157	14.58	0.49	Ester (Gatta et al., 2019)
Ethene, methoxy-	C_3H_6O	58	15.76	1.06	Poly vinyl methyl ether (Yang et al., 2018)
Tetradecanoic acid (Myristic acid)	$C_{14}H_{28}O_2$	228	16.26	1.42	Saturated long-chain fatty acid with a 14-carbon backbone (Youssef et al., 2011)
6- Dodecanone	$C_{12}H_{18}O$	178	16.80	0.73	Ketones (Zheng et al., 2019)
Hexadecan-2-one	$C_{16}H_{32}O$	240	16.94	1.08	Ketones (Zhang, 2020)
3,7,11-Trimethyl-1-dodecanol	$C_{15}H_{32}O$	228	17.05	0.34	Fatty alcohol (Al-Wathnani, 2012)
1,4-diaza-2,5 dioxobicyclo[4.3.0 nonane]	$C_7H_{10}N_2O_2$	154	17.95	2.27	(Raharja et al., 2019)
Pentadecanoic acid	$C_{15}H_{30}O_2$	242	18.25	9.36	Saturated fatty acid (Youssef et al., 2011)
Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl(-	$C_{11}H_{18}N_2O_2$	210	19.91	21.67	Heterocyclic compounds with nitrogen hetero-atom(s) (Kiran et al., 2018)
Hexadecanoic acid	$C_{16}H_{32}O_2$	256	20.35	1.89	Palmitic Acid is a saturated long-chain fatty acid. (Rajaofera et al. 2019)
Heptadecanoic acid	$C_{17}H_{34}O_2$	270	21.32	1.09	Fatty acid (Mohamad et al., 2018)
9-Octadecenoic acid (Z)-	$C_{18}H_{34}O_2$	282	21.47	2.69	Fatty acid (Mohamad et al., 2018)
cis-Vaccenic acid	$C_{18}H_{34}O_2$	282	23.04	1.26	Fatty acid

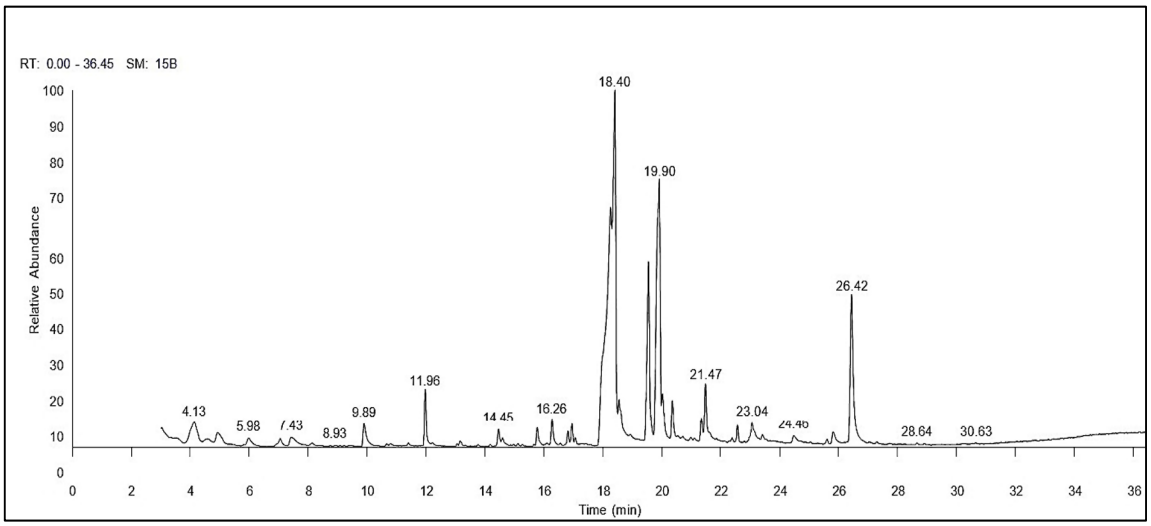

Fig. 8. GC–MS of *B. atrophaeus* ethyl acetate crude extract

Discussion

Controlling phytopathogens is an ongoing challenge because there is always a need for efficient approaches to treat infected plants. The commonly used approach is chemical control, but it has many disadvantages such as an adverse environmental and human health impact, a high cost, and inefficiency (Qing et al., 2015). Therefore, there is a continuous search for clean, eco-friendly, and safe alternatives to the microbicides currently used. One such eco-friendly alternative is biological control, where microbial antagonists with unique characteristics are used to inhibit phytopathogen growth (Berg et al., 2000).

In the present study, fifteen bacterial isolates were isolated from soil samples collected from different localities in Matrouh Governorate, Egypt. These isolates were screened for antibacterial activity using the well diffusion method (Wayne, 2011). Isolate SM3 was found to be the most antagonistic against four soft rotting bacterial strains, namely, *E. carotovora* subsp. *carotovora* strains Pep7C, Cab21B2, Cab45B and *E. chrysanthemi* strain Car1B. Molecular identification by 16S rRNA encoding gene sequence analysis identified isolate SM3 as *B. atrophaeus*.

Bacterial soft rot disease is one of the most destructive biotic diseases (Abu-Obeid, 2019), and soft rot erwinias, has been implicated in this disease (Abu-Obeeid et al., 2018; Wang et al., 2020). It has a wide host range and causes a great loss in many important crops (Charkowski, 2018).

Several studies have evaluated the effectiveness of different *Bacillus* spp. as a biocontrol agent against *E*cc. Chandrasekaran & Chun (2016) investigated the possible mechanisms of resistance induction in tomato plants infected with *Ecc.* They observed that the disease incidence was reduced by (36%) in plants treated with *B. subtilis* CBR05. Furthermore, the bacterial antagonist *B. amyloliquefaciens* BGP20 was found to be effective in controlling *E. carotovora* subsp. *carotovora* on green pepper and Chinese cabbage (Zhao et al., 2013).

Other members of the genus *Bacillus* have been isolated from the soil and showed *in vitro* antagonistic activity against different human and plant pathogenic microorganisms (Todorova & Kozhuharova, 2010; Abada et al., 2014).

In the present study, an *in vitro* experiment was conducted on agar plates to assess the antagonistic activity of *B. atrophaeus* isolate SM3 against four phytopathogenic fungal isolates. The results revealed that the antagonistic bacterium restricted the mycelial growth of all the tested fungi.

Additionally, the antifungal activity of *B. atrophaeus* isolate SM3 was evaluated in liquid medium and mycelial growth inhibition was also observed for all tested phytopathogenic fungi. However, the level of inhibition of fungal mycelial growth was higher in the liquid medium

than in the solid medium. This could be attributed to the increased chance of contact between the extracellular inhibitory metabolites and the fungal mycelium, while in agar plates, the diffusion of the produced inhibitory metabolites may be slower because they cannot easily diffuse through the agar medium.

The highest percentage of mycelial growth reduction was observed for *C. lunata* followed by *Penicillium* sp., *F. oxysporum* and *A. awamori*. Yuvarani et al. (2021) studied the antifungal activity of *Bacillus* spp. (BS3) against *C. lunata* which causes rice grain discoloration disease and observed inhibition of the pathogen's mycelial development by 48.8%. In monocotyledon crops like maize, *C. lunata* is most known for causing seedling blight and seed germination failure (Barupal et al., 2021). Moreover, it causes other diseases such as early blight of tomato (AbdElfatah et al., 2021). *Penicillium* sp., which is a common pathogen for post-harvest diseases, was found to be inhibited by the secondary metabolites of *B. velezensis* wr8 (Wang et al., 2022). Additionally, *B. thuringiensis* CHGP12 was reported as a promising strain against *Fusarium* wilt of chickpea, with high antagonistic and growth-promoting potential (Fatima et al., 2022).

B. atrophaeus was reported to act as biological control agent against other Phytopathogenic fungi including, *Botryosphaeria dothidea* that cause apple ring rot disease (Mu et al., 2020) and *Verticillium dahlia* the cause of wilt disease of some plants (Mohamad et al., 2018).

Phytopathogenic fungi treated with the antagonistic bacterium *B. atrophaeus* SM3 were examined using TEM. All examined isolates showed a distorted appearance as vacant mycelia with thinner and distorted cell walls. Similar structural changes have been reported when *B. atrophaeus* was used as a biological control agent against phytopathogenic fungi; *Colletotrichum nymphaeae* which causes strawberry anthracnose disease and *Alternaria alternata* which causes disease in tomato (Alijani et al., 2021; Chacon-Lopez et al., 2021). Cell wall distortion may be attributed to the fact that most of the pathogenic fungi that belong to the phylum Ascomycota and Basidiomycota have a cell wall comprising a mixture of chitin, glucan and mannoproteins (Vega & Kalkum, 2012) which can be lysed by lytic enzymes secreted by the antagonistic bacteria (Xu et al., 2014). Rajaofera et al. (2018) attributed the morphological changes occurred on fungal mycelium to bacterial secondary metabolites such as chitinases, proteases, siderophores, and others which suppressed fungal growth. Generally, *Bacillus* spp. secrete secondary metabolites with antifungal and antibacterial properties, as well as siderophores (Ajilogba & Babalola, 2019).

The capability of *Bacillus* spp. to inhibit the growth of a variety of bacterial and fungal isolates could be due to the production of many active compounds (Fernandes et al., 2007). GC-MS has been used to identify various bioactive metabolites that possess a range of antimicrobial activities. Screening of a new class of anti-microbial compounds is becoming increasingly vital for treating major infectious diseases that have developed from multidrug resistant bacteria (Kiran et al., 2018). GC-MS analysis of *B. atrophaeus* SM3 crude extract, detected seventeen bioactive compounds belonging to different classes of chemical compounds. One of the most interesting identified compounds is Tridecanoic acid (TDA) which is known for its antimicrobial activity, especially in extracts from *Bacillus* spp. (Chowdhury et al., 2021). Additionally, TDA has been reported to stabilize silver nanoparticles, leading to substantially better antibacterial effectiveness against the target pathogens, and this could be used to control plant and human diseases (Chowdhury et al., 2021).

Bacteria of the genus *Bacillus* are considered microbial factories that produce many physiologically active chemicals that are capable of inhibiting the growth of a variety of pathogenic bacteria and fungi (Prajakta et al., 2019). Surfactin is one of the lipopeptides produced by *Bacillus* spp., and its hydrophobic pentadecanoic fatty acid side chain seems to be the most active with regard to its hemolytic activity (Deleu et al., 2003). Pentadecanoic acid was recorded as one of the chemical compounds in the crude extract of *B. atrophaeus* isolate SM3 which might enhance its antimicrobial activity. Cell-free extracts of *B. subtilis* strain OKB105 have been found to produce lipopeptide biosurfactants in presence of L-amino acids, myristic acid, coenzyme A, ATP, and H_2O_2 (Youssef et al., 2011). The main bioactive metabolite detected in the crude extract of *B. atrophaeus* SM3 at the highest level is Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-

3-(2-methylpropyl), which has been reported to function as an antibiotic, as well as an antitumor, antifungal, anti-inflammatory and cholesterol reducing drug (Kiran et al., 2018). *B. tequilensis* displayed promising antimicrobial activity against multidrug resistant *Staphylococcus aureus* due to the presence of pyrrole as its main bioactive metabolite (Kiran et al., 2018). Fatty acids have long been known as antimicrobial agents, and various fatty acids are identified in *B. atrophaeus* SM3 crude extract such as Heptadecanoic acid and 9-Octadecanoic acid (Z). Casillas-Vargas et al. (2021) reported that both saturated and unsaturated fatty acids seemed to possess antimicrobial properties against multidrug-resistant bacteria. Moreover, they found that fatty acids could be used in combination with other antibiotics (synergistically) to increase their effectiveness against pathogenic microorganisms. Another study reported that the lipopeptides of *B. atrophaeus* CAB-1 suppressed cucumber powdery mildew and tomato gray mold activity and produced different volatile compounds such as alcohols, phenols, amines, and alkane amides (Ajilogba & Babalola, 2019). Metabolites produced by *B. atrophaeus* B5 were reported to exhibit antifungal activity against *Alternaria alternata* and reduce the level of spore germination (Chacón-López et al., 2021). GC-MS analysis of *B. atrophaeus* SM3 crude extract also revealed the presence of 1, 4-diaza-2, 5-dioxo-3-isobutyl bicyclo[4.3.0]nonane which has been reported as an antibacterial compound against biofilm forming *Klebsiella pneumoniae* by decreasing its metabolic activity and modifying exopolysaccharide production of the biofilm forming cells at a concentration of 300µg/mL (Nadar Rajivgandhi et al., 2020).

Conclusion

Analysis of 16S rRNA encoding gene sequences of the antagonistic soil bacterial strain SM3 revealed that it belongs to *B. atrophaeus* (GenBank accession no.: MZ741525). This strain, out of 15 isolates, showed the highest antagonistic activity against four soft rotting bacterial isolates, *Ecc* isolates, Pep7C, Cab21B2 and Cab45B and *E. chrysanthemi* strain Car1B as well as four fungal phytopathogens, *C. lunata*, *F. oxysporum*, *A. awamori* and *Penicillium* sp. Moreover, the mycelial mass of these fungal isolates was reduced when grown in potato dextrose broth in presence of *B. atrophaeus* SM3. TEM examination of the fungal isolates antagonized by this antagonistic bacterium showed different morphological changes including distortion of the cell wall, a folded and light scattering cytoplasm, and cytoplasmic reduction. GC-MS mass spectroscopy analysis of the crude extract of *B. atrophaeus* SM3 revealed the presence of Tridecanoic acid (TDA), Pyrrole, Pentadecanoic acid and different types of fatty acids. Almost all the identified compounds are known as biological agents for controlling pathogenic microorganisms, suggesting that *B. atrophaeus* SM3 could be used as an eco-friendly biocontrol agent for phytopathogenic microorganisms.

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النشاط العدائي لبكتريا *Bacillus atrophaeus* (MZ741525) ضد بعض الكائنات الدقيقة الممرضة للنبات

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تم عزل 15 عزلة بكتيرية من عينات تربة تم تجميعها من محافظة مطروح مصر، كما تم اختبار قدرتها علي تثبيط نمو بعض العزلات البكتيرية والفطرية الممرضة للنبات. وقد أظهرت العزلة MS3 أعلى تأثير مثبط للكائنات المختبرة. وتم تعريفها باستخدام تحليل التسلسل الجيني عن طريق 16S rRNA encoding gene sequence analysis على أنها سلالة *Bacillus atrophaeus* SM3 (رقم التسجيل في بنك الجينا MZ741525). تم تأكيد نشاطها المضاد تجاه أربع عزلات بكتيرية مسببة لمرض العفن الرخو للنبات وهي: *Erwinia carotovora* subsp. *carotovora*) سلالات Pep7C، و Cab21B2، وCab45B و Erwinia chrysanthemi Car1B) وأربع عزلات مسببة للأمراض الفطرية للنبات و هي *Curvularia lunata*، *Fusarium oxysporum*، *Aspergillus awamori* و *Penicillium* sp.. وقد استطاعت السلالة البكتيرية *B. atrophaeus* SM3 تثبيط نمو العزلات الفطرية: *C. lunata* بنسبة 93.4٪ ، يليها *Penicillium* sp. (77.8٪)، *F. oxysporum* (61.7٪) ، و *A. awamori* (41.0٪). هذا وقد تم فحص العزلات الفطرية المعالجة بالسلالة *B. atrophaeus* SM3 بواسطة الميكروسكوب الإلكتروني الناقل والذي أسفر عن تغيرات مورفولوجية، متضمنة انكماش الخلية، وكذلك لوحظ اختلاف في سمك جدار الخلية، والسيتوبلازم، واختزال سيتوبلازمي. كما تم استخلاص المواد الفعالة من بكتيريا *B. atrophaeus* وتعريفها بواسطه بواسطة التكليل الطيفي الكتلي وأظهرالتحليل وجود العديد من المركبات، مثل حمض tridecanoic، البيرول، حمض البنتاديكانويك pentadecanoic، والأحماض الدهنية المختلفة، ذات النشاط البيولوجي المعروف في مقاومة الكائنات الحية الدقيقة المسببة للأمراض. وعلي ذلك، فإن نتائج هذه الدراسة تبين أن هذه البكتريا السلالة البكتيرية قد يكون لها دور واعد في مجال المكافحة الحيوية للأمراض الميكروبية للنبات والذي يعد من الطرق الصديقة للبيئة ذات المردود الإيجابي علي البيئة وعلي الصحة العامة للإنسان.