Research Article

Antifungal Activity of *Moringa oleifera* Leaf Extract and *Bacillus subtilis* Product Against *Rhizoctonia solani*, the Causal Agent of Tomato Root Rot Disease in Egypt

Rasha E. Selim[1], Sahar. M. Moussa[2] and Hanan F.B. Youssef[3]*

[1]Agricultural Research Center, Central Agricultural Pesticides Laboratory(CAPL), El- Sabaheya, Alexandria, Egypt; [2]Agricultural Research Center, Sugar Crops Research Institute, Breeding and Genetics Department, Alexandria, Egypt; [3]Bacterial Diseases Research Department, Plant Pathology Research Institute, Agricultural Research Center, El-Sabaheya, Alexandria, Egypt.

Abstract | *Rhizoctonia* root rot is one of the most destructive diseases of tomato and other crops. This study aimed to investigate antifungal efficacy of *Moringa oleifera* leaf methanol extract, *Bacillus subtilis* product (sting), and their combination against the fungal causal agent of tomato root rot disease. *M. oleifera* extract is considered as a biofungicide against plant pathogenic fungi. *B. subtilis* represents a promising and more effective biocontrol and growth-promoting agent for tomato plants, regarded as an ecofriendly and sustainable tool in modern agriculture. In this study, the antifungal activity of *M. oleifera* extract and *B. subtilis* sting against *Rhizoctonia* root rot was investigated under laboratory and greenhouse conditions. Leaf extract of *M. oleifera* was analyzed by Gas chromatography-mass spectrometry (GC-MS). The main extract constitutes were hexadecanoic acid (9.53%), hexadecanoic acid ethyl ester (10.19%), and 9,12,15-octadecatrienoic acid ethyl ester (10.09%). *In vitro* assay *M. oleifera* extract achieved 85% inhibition against *Rhizoctonia solani* at the higher concentration of 3000 µg/ml; however, when combined with *B. subtilis* sting at a recommended dose of 6000 µg/ml, the inhibitory effect reached 95.33%. *B. subtilis* sting individually achieved 69.2% inhibition at a recommended dose of 6000 µg/ml. The interactions among *M. oleifera* extract, *B. subtilis* sting, their combination, and *R. solani* were evaluated through scanning electron microscopy (SEM). Ultrastructural effects were studied and the results indicated that *M. oleifera* extract caused deformities with shriveling and abnormal coiling of pathogen's hyphal filaments. *B. subtilis* sting caused shrinkage, coiling, hyphal lysis, and breakage, while their combination caused hyphal breakdown, fragmentation, and hyphal lysis. *M. oleifera* extract at 2000 µg/ml reduced disease severity by 62.79%, while *B. subtilis* sting at 6000 µg/ml achieved a remarkable suppression. The combined treatment was most effective, reaching 88% relative disease control (RDC) and enhanced growth parameters, including dry weight of shoot and root. Biochemical analyses showed that the combination treatment maximized defense responses by increasing polyphenol oxidase (PPO) activity by 67.15–65.82%, peroxidase (POD) by 78.18–70.45%, total phenols reached 5404.12 µg tannic acid/g fresh leaves, and recorded total soluble proteins of 280.07 mg/g fresh leaves. These findings indicate that integrating *B. subtilis* sting with *M. oleifera* extract provides a sustainable, effective strategy for disease control and growth promotion in tomato.

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***Correspondence** | Hanan F.B. Youssef, Bacterial Diseases Research Department, Plant Pathology Research Institute, Agricultural Research Center, El-Sabaheya, Alexandria, Egypt; **Email:** hananfarouk83@yahoo.com
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Introduction

Tomato (*Lycopersicum esculentum L.*) is considered as one of the most important vegetable crops in the world due to its nutritional value and widespread consumption. Globally, tomato ranks as the second most consumed vegetable crop, following the potato. It belongs to the *Solanaceae* family, which includes many agriculturally and economically important plants. Tomatoes are rich in bioactive compounds, such as tomatine and tryptophan, which play a crucial role in human nutrition and health by contributing to essential metabolic processes. In terms of cultivated area among processed vegetables, tomato occupies the largest global share, followed by potato (Behiry *et al*., 2023). Tomato plants are highly susceptible to a wide variety of phytopathogens, including fungi, bacteria, nematodes, and viruses. These pathogens may lead to severe diseases that substantially reduce crop yield and quality. Among the soil-borne fungi, *Fusarium oxysporum* (Schltdl, 1824), *Rhizoctonia solani* (Kühn, 1858), *Verticillium* spp. (Nees, 1816), and *Pythium* spp. (Pringsh, 1858) are well-documented as major causal agents of root rot in tomatoes (Singh *et al*., 2017). *R. solani* is considered as one of the most aggressive and destructive fungi, causing serious root diseases, damping-off, and seedling mortality in tomato cultivations. Infection by *R. solani* is manifested by various symptoms, including root decay, seedling collapse, and reduced plant vigor, which collectively lead to considerable losses in commercial production (Channa *et al*., 1995). Chemical control using fungicides has traditionally been the most widely applied approach for managing these fungal diseases. However, frequent application of fungicides has led to the emergence of resistant pathogen strains, limiting the overall efficacy of chemical treatments. In addition to resistance development, extensive use of chemical fungicides poses potential hazards to the human health and the environment, including soil and water contamination, toxic residues in crops, and disruption of beneficial microorganisms in agricultural ecosystems (Lahlali *et al*., 2022; Behiry *et al*., 2023). These challenges have necessitated the exploration of environmentally friendly and sustainable alternatives that can provide effective disease management while minimizing adverse effects. One of the promising alternatives is the use of plant-derived extracts and oils. *Moringa oleifera* commonly known as moringa, has recently gained attention due to its potential applications in agriculture as a natural biofungicide and growth-promoting agent. *M. oleifera* leaves are rich in bioactive compounds, including phenols, flavonoids, and alkaloids, exhibiting appreciable antibacterial and antifungal properties. Several studies have highlighted the potential of *M. oleifera* extracts as an eco-friendly strategy to control plant pathogens while reducing reliance on synthetic chemicals (Khedr *et al*., 2022; Ahmed *et al*., 2024). Another sustainable approach for disease management involves the use of beneficial microorganisms as biological control agents. Microorganisms such as *Trichoderma album* (Preuss, 1851) and *Bacillus megaterium* (de Bary, 1884) when applied as cell suspensions or culture filtrates, have been reported to inhibit the growth of plant pathogens through multiple mechanisms such as parasitism, antibiosis, and competition for nutrients and space (Ashry *et al*., 2022; El-Saadony *et al*., 2022). Among these biocontrol agents, *B. subtilis* has been extensively studied due to its strong ability to suppress fungal diseases in tomato and other crops. The effectiveness of *B. subtilis* is attributed to its rapid colonization of plant surfaces, preventing fungal spores from reaching stomatal openings and establishing an infection. In addition, *B. subtilis* competes intensively with pathogens for essential nutrients and oxygen, effectively limiting pathogen growth. Furthermore, *B. subtilis* produces extracellular enzymes such as β-1,3-glucanase and proteases, which degrade fungal cell walls, directly inhibiting pathogen development (Asaka and Shoda, 1996; Sehsah *et al*., 2022).

The present study was designed to evaluate the effectiveness of *M. oleifera* leaf extract, *B. subtilis* (sting), and their combined application against *R. solani* infecting tomato plants, as ecofriendly and sustainable alternatives to chemical pesticides. Additionally, this study investigated the activities of key oxidative enzymes, including peroxidase (POD) and polyphenol oxidase (PPO), and the levels of total soluble phenols and total soluble proteins to elucidate the underlying mechanisms through which each treatment exerts its antifungal impact. This comprehensive approach aimed to provide insights into alternative disease management strategies that are effective and ecofriendly, contributing to sustainable tomato production and reduced reliance on chemical fungicides.

Materials and Methods

Preparation and maintenance of fungal inoculum

A pathogenic strain of *Rhizoctonia solani* (GenBank

Accession No. PQ721626) was obtained from the Fungicide Bioassay Laboratory, Central Agricultural Pesticides Laboratory (CAPL), Agricultural Research Center, El- Sabaheya, Alexandria, Egypt. The strain was maintained throughout the study on potato dextrose agar (PDA) medium and incubated at 25 °C.

Preparation and maintenance of Bacillus subtilis

Bacillus subtilis was supplied as a sting® 1.5% wettable powder (WP) (containing 1 × 10^8 cells/g of product) and manufactured by Trade Line Corporation, India.

Preparation and extraction procedure of Moringa oleifera leaves

Dried *M. oleifera* leaves were provided by the National Research Center, Dokki, Cairo, in collaboration with the Egyptian Scientific Society of *Moringa* (ESSM). For extract preparation, 200 g of dried leaf powder were immersed in 1000 ml of methanol in a 1-liter conical flask and subjected to mechanical shaking for 7 d to ensure efficient extraction. The resulting macerate was filtered using Whatman No. 1 filter paper. Solvent evaporation was carried out using a rotary evaporator (Heidolph Laborota 4000, Heidolph Instruments, Germany) to concentrate the crude extract. The crude extract was purified to remove undesired impurities by passing it through a 0.45 µm syringe filter (Sartorius®) as described by Ahmadu *et al.* (2020). Extraction yield was determined following the formula reported by Mushore and Matuvhunye (2013):

$$Yield\ (\%) = (Weight\ of\ crude\ extract / Weight\ of\ dried\ leaves) \times 100$$

Identification of bioactive compounds using GC–MS

The chemical composition of *M. oleifera* extract was analyzed using Thermo Scientific™ Trace™ 1300 series GC-TSQ Mass Spectrometer (Thermo Scientific, Austin, TX, USA) equipped with a TG–5MS capillary column (30 m × 0.25 mm × 0.25 µm). Approximately 1 µl of methanol diluted extract was injected automatically in split mode with a 4-min. solvent delay. The column oven temperature was programmed from 50 °C to 250 °C at 5 °C/min., held for 2 min., increased to 300 °C at 30 °C/min., and maintained for 2 min. The injector and transfer line temperatures were 270 °C and 260 °C, respectively, and helium was used as the carrier gas at 1 mL/min. The mass spectrometer operated in full scan mode with electron impact ionization at 70 eV, covering m/z 50–650, and the ion source temperature was set at 200 °C. Compound identification was achieved by comparing mass spectra with the NIST 14 and WILEY 09 libraries (Mamoun, 2016). This methodology provided a reliable profile of the major bioactive constituents present in the leaf extract.

Antifungal potential of Moringa oleifera, Bacillus subtilis and their combination on Rhizoctonia solani in vitro

The antifungal activity of *M. oleifera* extract, *B. subtilis* (sting) at a recommended sting product dose of 6000 µg/ml, and their combination were evaluated against *R. solani in vitro*. Seven concentrations of methanol *M. oleifera* extract and untreated control were incorporated individually into PDA plates using the poisoned food technique (Kumar *et al.*, 2008). A 5 mm mycelial disc cut from a 7-day-old culture of *R. solani* using a sterile cork borer was aseptically placed at the center of each plate and incubated at 25 °C. Treatments were replicated three times in a completely randomized design (CRD). After 7 d of incubation, radial fungal growth was measured in mm using a calibrated ruler and the percentage of inhibition was recorded according to Pandey *et al.* (1982) using the following equation.

$$\%\ Inhibition = [(DC - DT) / DC] \times 100$$

Where; C and T represent radial growth diameter in the control and treated plates, respectively. IC_{50} values were determined using linear regression analysis (Finney, 1971).

Scanning electron microscopy (SEM) examination

For microscopic visualization of the inhibition of *R. solani* growth induced by *M. oleifera* extract, *B. subtilis* sting, and their combination at the concentrations of 3000 µg/ml, 6000 µg/ml, and (3000 µg/ml + 6000 µg/ml), respectively, PDA agar plates were prepared. A line of *M. oleifera* extract was streaked at the center of the plate using a sterilized loop, two mycelial discs (5 mm in diameter each) of an activity growing culture of *R. solani* were added on both sides of the line at contrast distance opposite to the other edge of the plate. The same trend was made with *B. subtilis* sting and their combination mixture. After 5 d of incubation, scanning electron microscopy (SEM) examination of the culture plate was done according to Tahmasebi *et al.* (2015). Images were captured to visualize the interaction of the individual compounds with *R. solani*. A culture plate of *R. solani* served as control treatment.

Antifungal efficacy of Moringa oleifera extract, Bacillus subtilis and their combination against R. solani infecting tomato plant under greenhouse conditions

In vivo study

The *in vivo* antifungal potential of *M. oleifera* extract, *B. subtilis* sting, and their combination was evaluated against *R. solani* under controlled greenhouse conditions (28 ± 2 ˚C, 80–90% relative humidity, 12 h photoperiod). A pot experiment was conducted to assess the disease suppression and plant growth promotion. Steam sterilized soil was filled into 20 cm plastic pots, which were pre-inoculated with *R. solani*. Four-week-old tomato seedlings (*var*. Peto 86) were transplanted into these pots. Fungal inoculum was prepared by inoculating 500 g of pre-autoclaved wetted barley grains with two 5 mm mycelial discs of *R. solani* and incubated for 7 d at 25 ± 2 ˚C. The colonized grains were then air-dried, ground into a fine powder, and incorporated into the soil at a rate of 10 g/kg, applied 48 h before transplanting near the root and crown of each seedling.

Seven treatments, each with five replicate were applied as follows: G1, untreated control; G2, inoculated with *R. solani*; G3, infected plants treated with *B. subtilis* sting at recommend dose 6000 µg/ml; G4, infected plants treated with 1000 µg/ml *M. oleifera* extract; G5, infected plants treated with 2000 µg/ml *M. oleifera* extract; G6, infected plants treated with 1000 µg/ml *M. oleifera* extract combined with *B. subtilis* sting at 6000 µg/ml; and G7, infected plants treated with 2000 µg/ml *M. oleifera* extract combined with *B. subtilis* sting at 6000 µg/ml. Extract concentrations were selected based on IC_{50} values obtained from preliminary *in vitro* assays. Thirty day after transplantation, leaf samples were collected to assess the defense-related responses, including polyphenol oxidase (PPO), peroxidas (POD), total phenolic compounds, and total soluble proteins. Moreover, root disease severity was evaluated using a 0–5 scale based on the extent of root browning (Abdeljalil *et al*., 2016; Heflish *et al*., 2021), where 0 = no symptoms, 1 = 0–25% browning, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%, and 5 = plant death. The disease severity index (DSI) was calculated according to Cohen and Mosinger (1991).

$$DSI\ \% = ((n \times c)/N \times df)$$

Where N is the total number of plant, c is the number of categories, n is the total number of infected roots for each category, and df is the degree of freedom.

Relative disease control percentage (RDC %) was calculated based on disease severity index according to Abbasa *et al*. (2019) as the following equation.

$$RDC\ (\%) = [(DSI\ control - DSI\ treatment) / DSI\ control] \times 100$$

In addition, plant growth parameters, including dry weight of shoots and roots were measured to evaluate the effects of the applied treatments on overall plant performance.

Biochemical studies

Enzyme extraction: One gram of fresh tomato leaves was homogenized in 10 ml of sodium phosphate buffer (0.1 M, pH 7.5) containing 0.2 M sodium metabisulfite and sodium chloride. The homogenate was centrifuged at 11,180 ×g for 15 min at 4˚C. The supernatant was used to estimate the enzymes activity (Tuzun *et al*., 1989).

Polyphenol oxidase (PPO) activity:

The PPO activity was determined in reference to Gauillard *et al*. (1993). The reaction mixture consisted of 0.2 ml enzyme extract and 2.8 ml catechol solution (0.1 M) prepared in phosphate buffer (0.1 M, pH 6.8). A spectrophotometer Turner Model 390 (Turner Designs, USA) was used to measure the absorbance of the treatment and control samples at 575 nm. The activity was expressed as a percentage of control. Each value was reported as the average of three replicate.

$$Activity\ (\%) = (A1/A2) \times 100$$

Where; A1 is the absorbance of the treatment sample and A2 is the absorbance of the control sample.

Peroxidase (POD) activity:

POD activity was assessed using the previous methodology conducted by Murage and Masuda (1997) with some modifications. The reaction mixture consisted of 0.2 ml enzyme extract, 1.5 ml of 20% H_2O_2, and 1.5 ml catechol (0.04 M). The initial rate of rise in absorbance was determined within 1 min. at 470 nm using a spectrophotometer Turner Model 390 (Turner Designs, USA). Each value was presented as the average of three replicate.

$$Activity\ (\%) = ((A1/A2) \times 100)$$

Where; A1 is the absorbance of the treatment sample and A2 is the absorbance of the control sample.

Total soluble phenols were determined according to Slinkard and Singleton (1977) with slight modifications. A weight (0.5 g) of fresh tomato leaves was immersed in 8 ml of 80% methanol and kept overnight at room temperature. The extract was filtered, and the filtrate was diluted to 10 ml, serving as a stock solution for subsequent analysis. 0.2 ml of the stock solution was mixed with 1.4 ml distilled water and 0.1 ml of 50% (1 N) Folin–Ciocalteu reagent. After at least 30 sec, 0.3 ml of 20% (w/v) sodium carbonate was added. The reaction mixture was allowed to stand for 30 min. at 40°C, and the absorbance was measured at 765 nm using a Turner spectrophotometer, Model 390 (Turner Designs, USA). Total soluble phenol content was standardized against tannic acid, and the absorbance values were converted to µg tannic acid/g fresh weight of tomato leaves. Each value represents the average of three replicate. The results were expressed as tannic acid equivalents according to the following formula:

µg tannic acid/g fresh weight = ((OD/K) × (10/0.2)/g sample)

Where; OD = absorbance at 765 nm and K= the extension coefficient =0. 0.01690 µg/ml.

Total protein content was determined according to the method reported by Bradford (1976) with slight modifications described by Dixon (1985). A weight of 0.25 g of fresh tomato leaves was added to 5 ml of acetone. The plant sample was then transferred to 5 ml of 1 N NaOH and heated at 85°C for 1.5 h, followed by filtration. An aliquot of 0.1 ml of protein solution was mixed with 1 ml of Bio-Rad assay dye (prepared by diluting 1 ml of Bio-Rad dye in 5 ml of distilled water). The developed color was measured at 595 nm using a Turner spectrophotometer, Model 390 (Turner Designs, USA). Protein content was calculated from a standard curve prepared using known concentrations of Bovine Serum Albumin (BSA). Each value represents the average of three replicate.

mg protein/g fresh weight = ((OD/K)×100)/g sample

K= 0.029 mg/ml, and OD= The absorption at 595 nm

Statistical analysis

ANOVA for three-way Randomized Blocks was used to statistically analyze the collected data using MSTAT version 4 (1987). To ascertain whether the means of the different treatments differed significantly, the Least Significant Differences (LSD) test at $p \leq 0.05$ was employed.

Results

Identification of bioactive compounds using GC–MS

Table 1 and Figure 1 demonstrate the GC–MS analysis of *M. oleifera* extract that revealed a diverse profile of bioactive compounds. The major identified constituents included hexadecanoic acid (9.53%), hexadecanoic acid ethyl ester (10.19%), and 9,12,15-octadecatrienoic acid ethyl ester (10.09%).

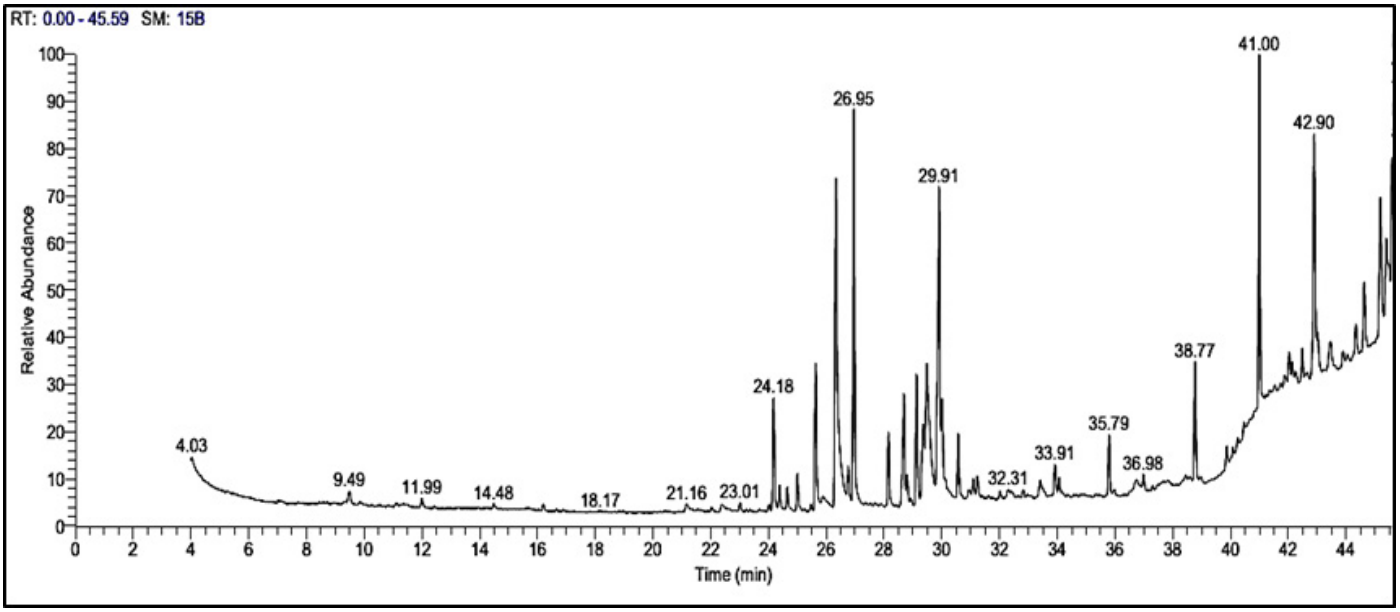


Figure 1: *GC-MS chromatogram of M. oleifera leaf methanol extract.*

Table 1: *Chemical composition of M. oleifera leaf methanol extract detected by GC–MS.*

Retention time (min)	Peak area (%)	Chemical name	Chemical class
24.18	3.00	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl	Fatty Alcohols and Isoprenoids
24.38	0.63	2-Methylhexadecan-1-ol	Fatty Alcohols and Isoprenoids
24.65	0.60	2-cis-9-Octadecenyloxyethanol	Fatty Alcohols and Isoprenoids
25.00	1.07	9-Icosyne	Alkyne
26.33	9.53	Hexadecanoate (Hexadecenoic acid)	Fatty acids
26.76	0.84	9-Hexadecenoic acid, ethyl ester	Fatty acid esters
26.95	10.19	Hexadecenoic acid, ethyl ester	Fatty acid esters
28.15	2.06	Trimethylsilyl palmitate	Fatty acid esters
28.68	3.27	Ethyl 9à-linolenate	Fatty acid esters
28.8	0.53	10-Octadecenoic acid, methyl ester	Fatty acid esters
29.12	3.67	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	Diterpene alcohol
29.36	1.68	Methyl 13,16-octadecadiynoate	Fatty acid esters
29.48	3.60	trans-13-Octadecenoic acid	Fatty acids
29.91	10.09	9,12,15-octadecatrienoic acid ethyl ester	Fatty acids
30.01	2.21	9-Octadecenoic acid (Z)-, ethyl ester	Fatty acid esters
30.57	1.75	Stearic acid, ethyl ester	Fatty acid esters
31.09	0.50	trans-13-Octadecenoic acid, trimethylsilyl ester	Fatty acids
31.24	0.63	(Z,Z)-1,3-dioctadecenoyl glycerol	Glycerolipids
33.41	0.59	3-Ethyl-3-hydroxyandrostan-17-one	Steroids and Sterols
33.91	0.93	1-methyl n-l-alpha-aspartyl-l-phenylalanate	Pharmaceuticals compounds
34.06	0.45	2,3-Dihydroxypropyl (9E)-9-octadecenoate	Fatty acid esters
35.79	1.66	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	Phthalate ester
36.98	0.41	8-(3-octyl-2-oxiranyl)octanoic acid	Oxygenated fatty acids
38.77	3.39	n-Heptacosane	Alkanes
39.86	0.60	2,3-Bis[(trimethylsilyl)oxy]propyl(9z,12z)-9,12-octadecadienoate	Glycerophospholipid derivatives (silylated
40.46	0.40	5-Fluoro ADB metabolite 7	Pharmaceutials compounds
40.99	8.84	n-Tetratetracontane	Alkanes
42.03	0.88	9,12-octadecadienoicacid(z,z)-,2,3-bis[(trimethylsilyl)oxy]propyl ester	Fatty acids

Other notable compounds were 2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-6-chromanol (7.43%) and tetratetracontane (8.84%). Overall, the identified compounds were classified into several chemical groups, with fatty acids representing the predominant class (24.10%), followed by fatty acid esters (22.93%), steroids and sterols (8.02%), terpenes (7.43%), fatty alcohols and isoprenoids (4.23%), and diterpene alcohols (3.67%). These chemical classes are widely recognized for their antimicrobial and antioxidant activities, providing a chemical basis for the observed bioactivity.

Effect of Morina oleifera extract, Bacillus subtilis (sting) and their combination on R. solani in vitro

The crude methanolic extract of *M. oleifera* leaves yielded 17.2%. *In vitro* assay presented in Figure 2 revealed that increasing concentrations of the extract considerably inhibited the radial growth of *R. solani*. Specifically, the extract achieved 85% inhibition at 3000 µg/ml, 63.33% at 2000 µg/ml, and 15.4% at 250 µg/ml. When combined with *B. subtilis* sting 6000 µg/ml (a recommended dose), the inhibitory effect of *M. oleifera* extract was enhanced, reaching 95.33% at 3000 µg/ml, 72.33% at 2000 µg/ml, and 19.67% at 250 µg/ml. *B. subtilis* sting achieved 69.2% inhibition at a recommended dose of 6000 µg/ml. In Table 2, the IC_{50} of the methanolic *M. oleifera* extract was evaluated as1045.65 µg/ml, indicating a relatively uniform sensitivity among the tested fungal population. These findings demonstrate that combined application of *M. oleifera* extract at 3000 µg/ml and *B. subtilis* sting

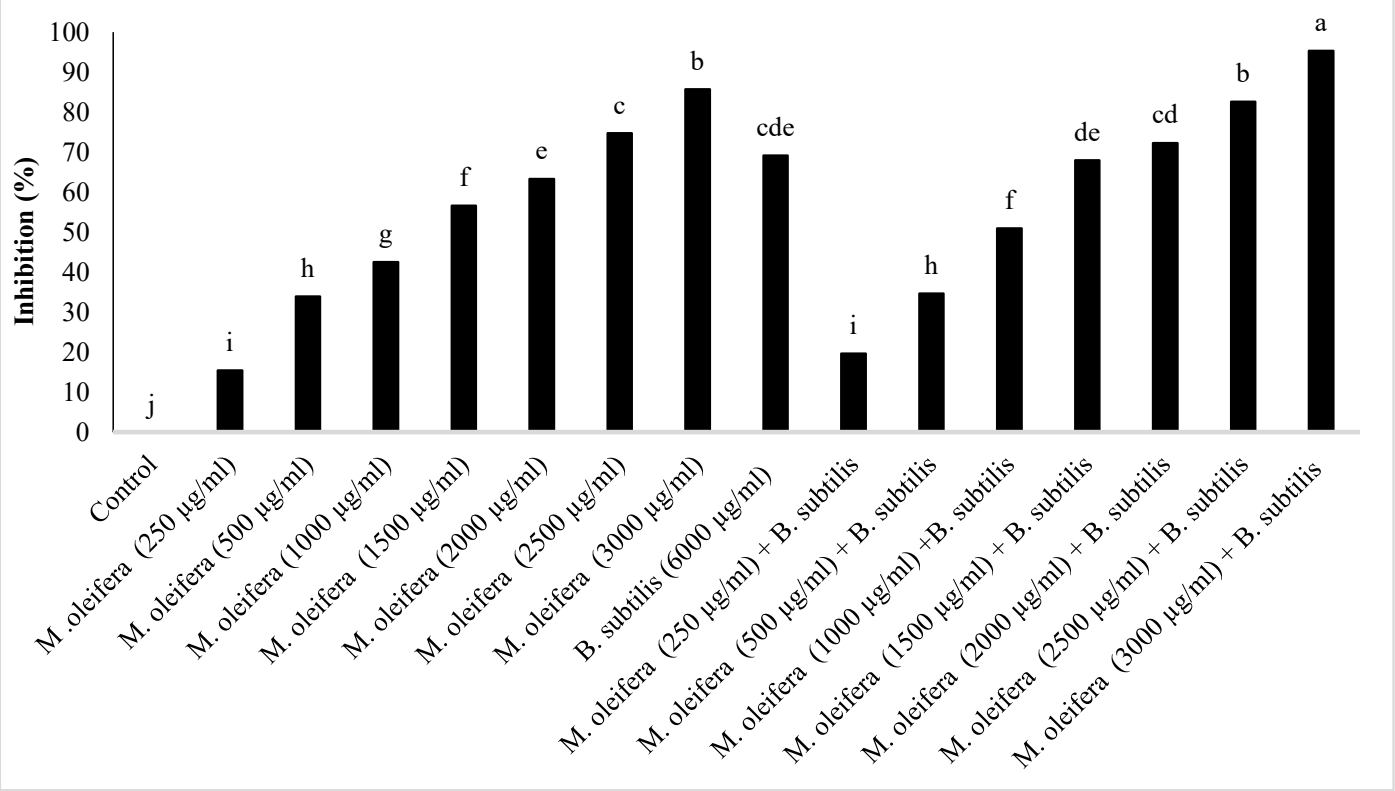


Figure 2: *In vitro effect of M. oleifera, B. subtilis (sting) at a recomended dose, and their combination on inhibition percentage of R. solani. Different letters denote significant changes among the treatments within the same bar, using the least significant difference test ($p \leq 0.05$).*

at 6000 µg/ml can achieve enhanced inhibitory effects, suggesting a synergistic potential effective for a biological control strategy.

Table 2: *Evaluation of IC_{50} of methanolic M. oleifera, leaves extract against R. solani.*

Treatment	IC_{25} (µg/ml)	IC_{50} (µg/ml)	Limit confidence		IC_{90} (µg/ml)	Slope* ± SE**
			Upper	Lower		
M. oleifera extract	421.82	1045.65	1195.52	906.58	5868.32	1.711± 0.14

Slope refers to the slope of the toxicity line, SE** refers to the standard error of slope (n=7 treatments).*

Scanning electron microscopy (SEM) examination

Interaction between Moringa oleifera extract and mycelia of *R. solani*: Scanning electron microscopy studies on the effect of *Moringa oleifera* extract on *R. solani* showing deformities with shriveling and abnormal coiling of hyphal filaments (Figure 3a1). lysis of hypha was occurred (Figure 3a2), loss of structural integrity of test fungus near the interaction zone, and The hyphal width of *R. solani* was greatly reduced (Figure 3a3) In contrast, hyphae of *R. solani* in control plates showed structural integrity with normal branching and a normal hyphal width (Figure 3d).

Interaction between Bacillus subtilis and mycelia of Rhizoctonia solani

Studies on the impact of *B. subtilis* sting on *R. solani* hyphae revealed coiling, hyphal lysis, and breakage (Figure 3b1), and deformation in hyphae occurred leading to their shrinkage (Figure 3b2), compared to that of healthy hyphae in control plates (Figure 3d).

Interaction between (a combination of Moringa oleifera extract + B. subtilis sting) and mycelia of Rhizoctonia solani

Breakdown and lysis of numerous hyphae of *R. solani* were observed (Figure 3c1), fragmentation and abnormal coiling of pathogen hyphae was noticed (Figure 3c2), and hyphal shriveling occurred (Figure 3c3) due to the inhibitory effect of *M. oleifera* extract + *B. subtilis* mixture, compared to the control (Figure 3d).

Efficacy of Moringa oleifera extract, Bacillus subtilis and their combination against R. solani infecting Tomato Plants in the greenhouse

Data presented in Figure 4A, B revealed that all treatments considerably reduced the percentage of tomato seedling root rot in the greenhouse trial. The disease severity index (DSI) was substantially declined while the relative disease control percentage (RDC%) rose,

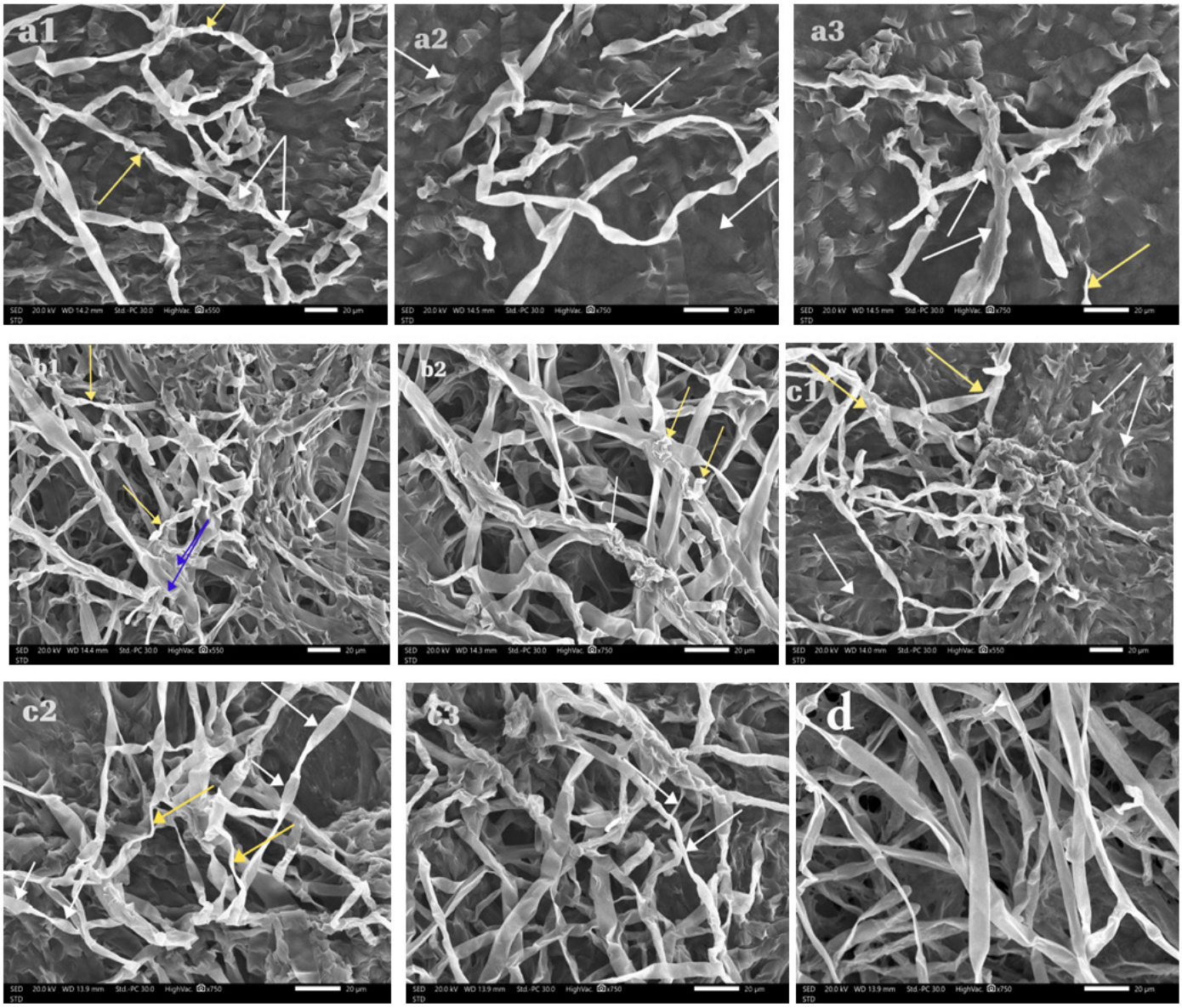


Figure 3: *Scanning electron micrographs of R. solani mycelia affected by M. oleifera extract, B. subtilis sting, and a combination of both. Interaction between R. solani and M. oleifera extract showing* ***(a1)*** *deformities with shriveling (white arrow) and abnormal coiling of hyphal filaments (yellow arrow), reduced hyphal width (yellow arrow),* ***(a2)*** *lysis of hypha (white arrow) and* ***(a3)*** *loss of structural integrity (white arrow) of tested fungal pathogen. Interaction between R. solani and B. subtilis sting showing* ***(b1)*** *coiling (yellow arrow), hyphal lysis (white arrow) and breakage (blue arrow), and* ***(b2)*** *deformation in hyphae (white arrow) and shrinking (yellow arrow). Interaction between R. solani and a combination of M. oleifera extract + B. subtilis sting showing* ***(c1)*** *hyphal breakdown (yellow arrow) and lysis (white arrow),* ***(c2)*** *hyphal fragmentation (white arrow) and abnormal coiling (yellow arrow), and* ***(c3)*** *hyphal shriveling (white arrow).* ***(d)*** *Normal hyphal branching and width in control plates.*

compared to the untreated control treatment. In this situation, *B. subtilis* sting at 6000 µg/ml considerably reduced the severity of root rot, recording RDC of 74.22% and DSI of 18.3%.

Upon combination of *M. oleifera* extract (2000 µg/ml) with *B. subtilis* sting (6000 µg/ml), DSI of 8.333 and RDC of 88.37% were recorded, indicating that this was the most effective treatment to reduce the root rot of tomato seedlings, followed by the combination of *M. oleifera* (1000 µg/ml) with *B. subtilis* sting (6000 µg/ml), recording DSI of 15 % and RDC of 79.79%. These results are encouraging because they exceeded the control rate obtained with these treatments. In the meantime, the fungicidal activity of *M. oleifera* extract was enhanced when *B. subtilis* sting and *M. oleifera* extract were combined in two concentrations. The results highlight the potent fungicidal activity of *M.*

oleifera extract, which was further amplified by synergistic interactions with *B. subtilis* sting, suggesting a promising strategy for controlling *R. solani* in the greenhouse.

Biochemical studies

The impact of Moringa oleifera extract, Bacillus subtilis and their combination on polyphenol oxidase and peroxidase activity

Figure 5A illustrates the impact of *M. oleifera* extract and its combination with *B. subtilis* sting on polyphenol oxidase (PPO) activity after 30 d of *R. solani* infection of tomato plants. Application of *B. subtilis* sting resulted in an increase in PPO activity by 60.395%, whereas the *M. oleifera* extract at 2000 and 1000 µg/ml enhanced the activity by 55.275% and 43.197%, respectively. However, when *M. oleifera* extract and *B. subtilis* sting were combined at the optimum dosages of *M. oleifera* extract (2000µg/ml) + *B. subtilis* sting (6000µg/ml) and *M. oleifera* extract (1000 µg/ml) + *B. subtilis* sting (6000 µg/ml), the activity of PPO enhanced by 67.147% and 65.819%, respectively.

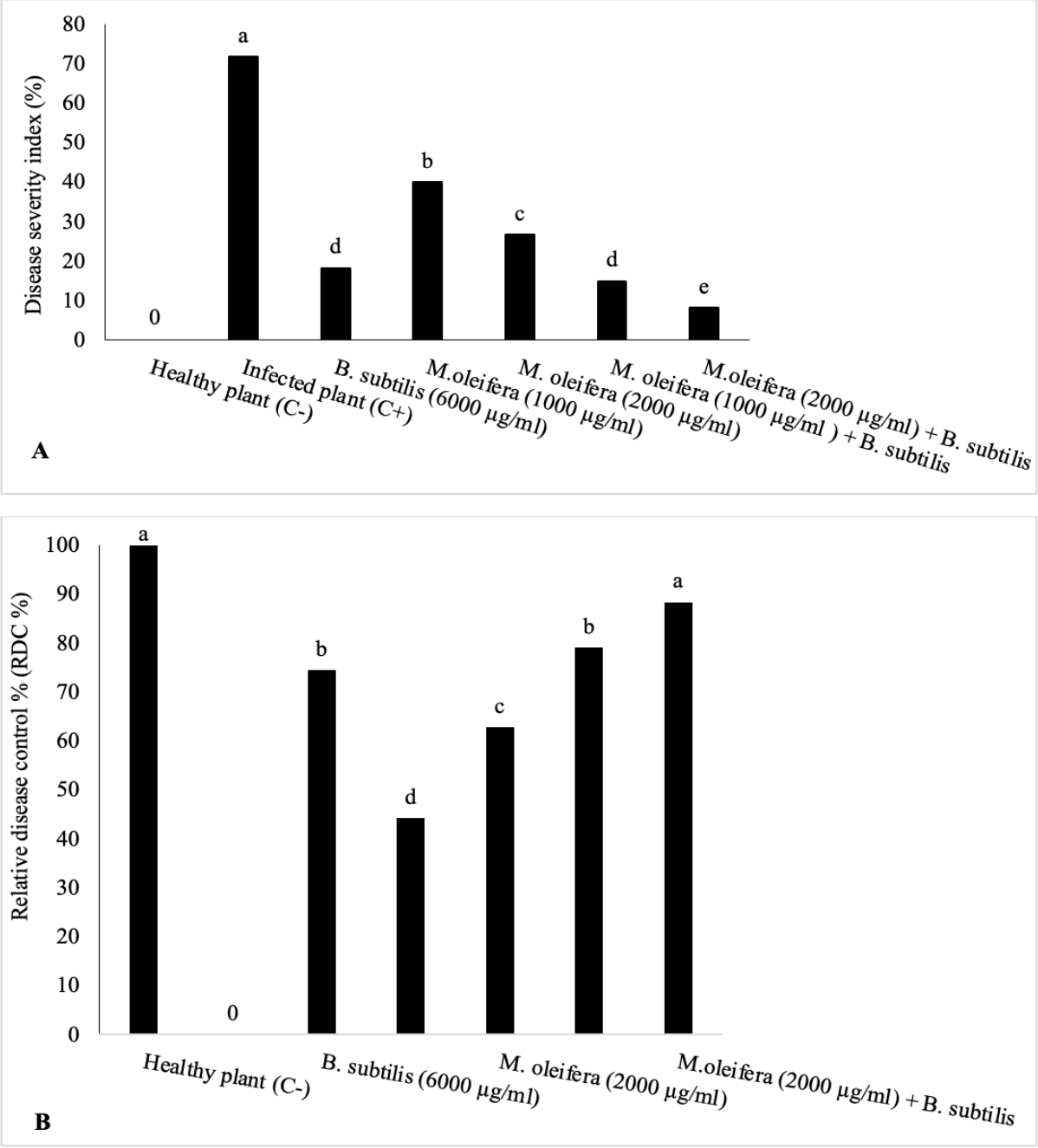
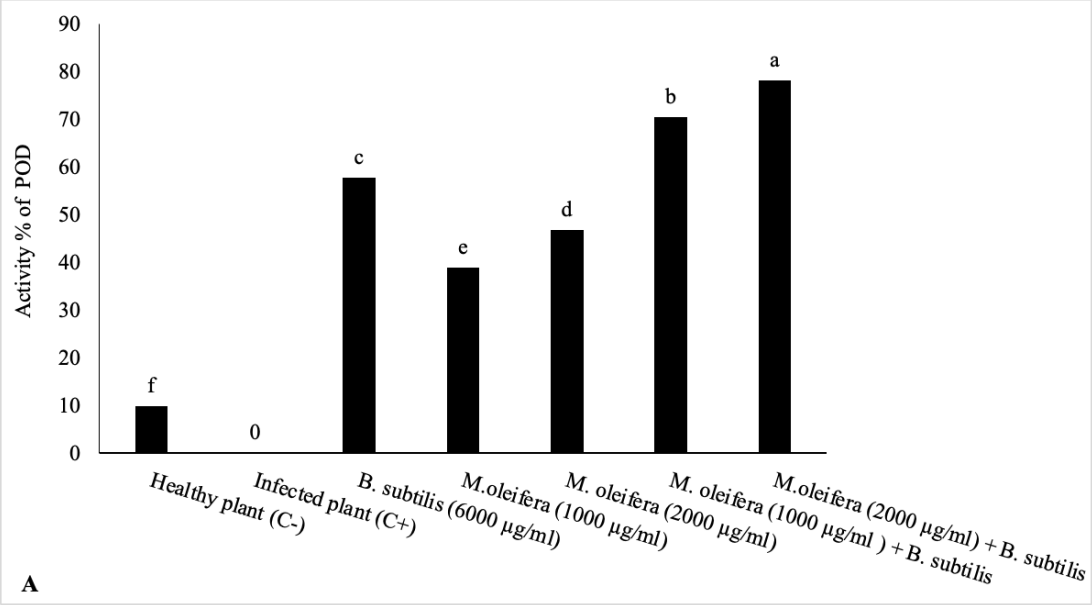


Figure 4: *(A) Disease severity index (DSI) of M. oleifera extract, B. subtilis and their combination against R. solani infecting Tomato Plants under greenhouse. Different letters denote significant changes among treatments within the same column, using the least significant difference test (p≤0.05). (B) Relative disease control % of M. oleifera_extract, B. subtilis and their combination against R. solani infecting tomato plants under greenhouse.*



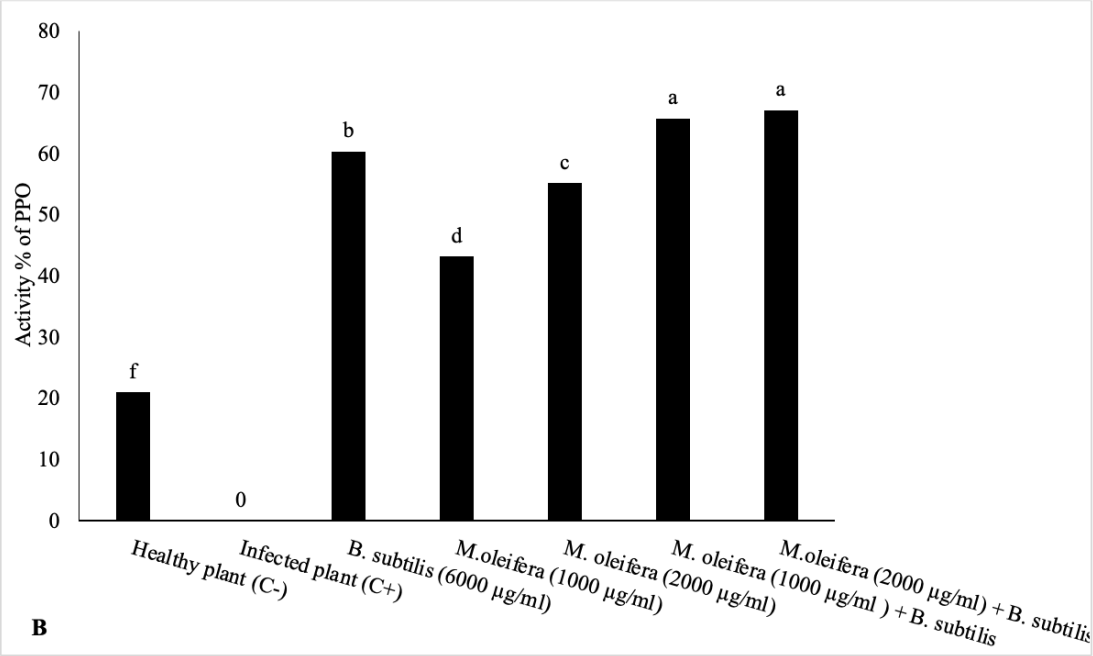


Figure 5: *The effect of M. oleifera, B. subtilis and their combination on polyphenol oxidase (PPO) (A) and peroxidase activity (POD) (B) in tomato plant leaves infected with R. solani. Different letters denote significant changes among treatments within the same column, using the least significant difference test (p≤0.05).*

After 30 d of *R. solani* infection of tomato plants, results presented on Figure 5B demonstrated that the use of *M. oleifera* extract and its combination with *B. subtilis* sting altered the activity percentage of peroxidase (POD). Application of *B. subtilis* sting enhanced POD activity by 57.747%, whereas the activity of POD at 2000 and1000 µg/ml of *M. oleifera* extract recorded 46.713% and 38.967%, respectively. However, the combination of *M. oleifera* extract (2000µg/ml) + *B. subtilis* sting (6000µg/ml) and *M. oleifera* extract (1000µg/ml) + *B. subtilis* sting (6000µg/ml) resulted in a remarkable increase in the POD activity by 78.183 and 70.446%, respectively.

Impact of Moringa oleifera extract, Bacillus subtilis and their combination on the total phenol (µg tannic acid /gm f.wt) and total soluble proteins (mg protein/gm f.wt) in leaves of tomato plant infected with Rhizoctonia solani

In the presence study, the contents of total phenol and total soluble proteins were increased remarkably in tomato leaves in all treatments, compared to the untreated control (Figure 6A).The best treatments involving the contents of total soluble phenol was recorded by *M. oleifera* extract (2000 µg/ml)+*B. subtilis* sting (6000µg/ml) with a value of 5404.12 µg tannic acid/g fresh weight of leaves of tomato, and the second place of increase was observed in seedlings

treated with *M. oleifera* extract (1000 µg/ml)+*B. subtilis* sting (6000µg/ml) recoding a value of 5111.383 µg tannic acid/g fresh leaves. However, *B. subtilis* sting, *M. oleifera* extract at 1000 and 2000 µg/ml recorded 4913.883, 4365.63, and 4676.93 µg tannic acid/g fresh leaves; respectively, compared to the control (3185.147. µg tannic acid/g fresh leaves). Results presented in Figure 6B demonstrate an appreciable increase in contents of total soluble proteins, particularly in *M. oleifera* extract at 1000 and 2000 µg/ml + *B. subtilis* sting, recording 222.457 and 280.067 mg protein/g of fresh leaves, respectively. There was no detected considerable difference between the two concentrations of *M. oleifera*, but *B. subtilis* sting displayed a remarkable increase of 203.310 mg protein/g of fresh leaves.

Impact of Moringa oleifera extract, Bacillus subtilis and a combination of both on the morphogenesis of tomato plants

Figure 7 presents the obtained data on the effects of adding *M. oleifera* extract, *B. subtilis* (sting), and a combination of both to tomato seedlings transplanted in *R. solani*-infested soil on the dry weight of shoots (Figure 7A) and roots (Figure 7B). The obtained results showed that the plants treated with *B. subtilis* (sting) had a higher dry weight of shoots and roots compared to all the other treatments. *B. subtilis* sting recorded the highest shoot dry weight (3.37 g), followed by the healthy plants (2.55 g), whereas the infected plants and those treated with *M. oleifera* extract (1000-2000 µg/ml) or in a combination with *B. subtilis* sting (6000 µg/ml) showed no considerable differences, where the shoot dry weight values ranged from 1.17 to 1.48 g.

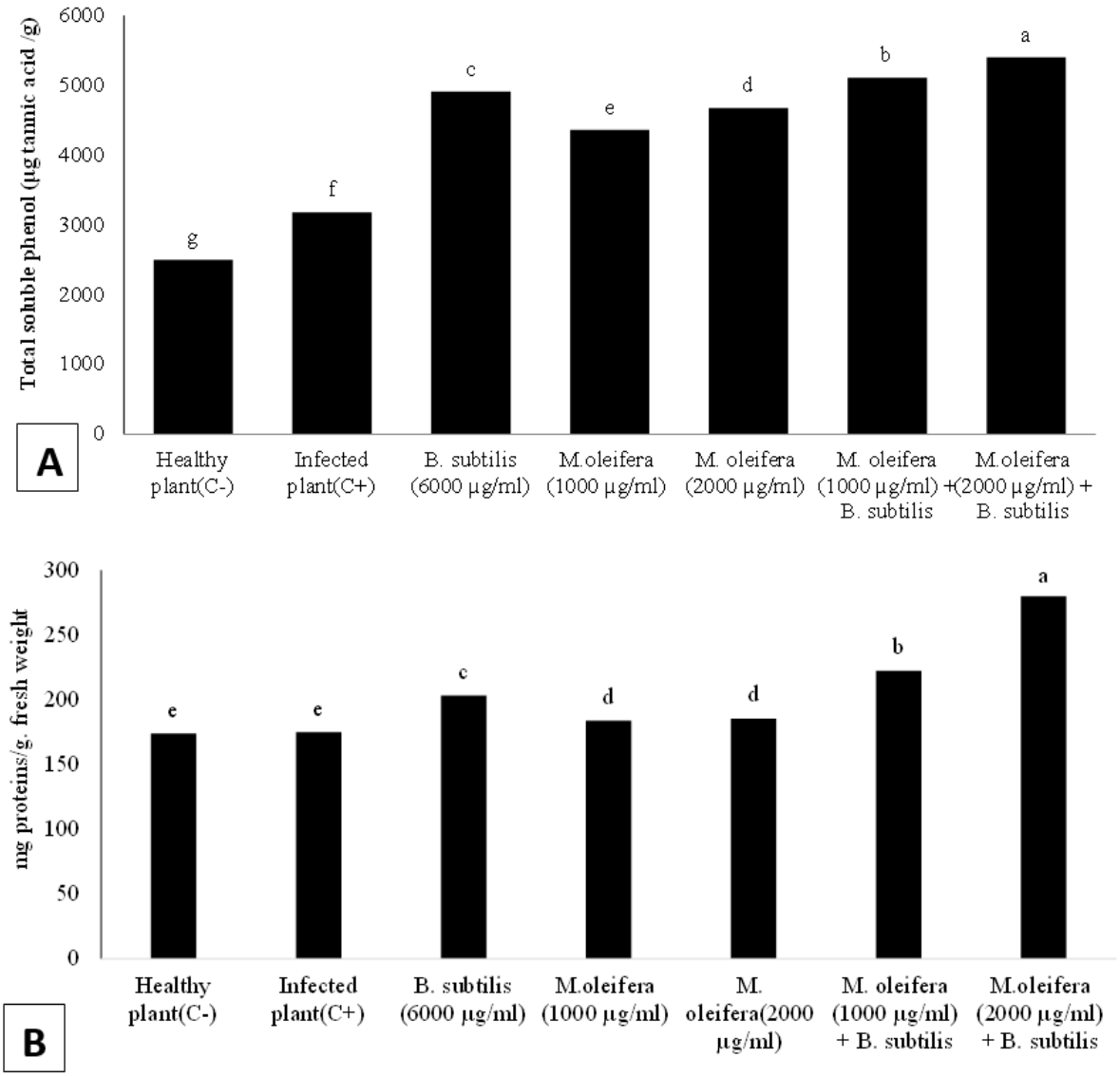


Figure 6: *Impact of M. oleifera extract, B. subtilis and their combinations on the content of the total phenol (A) and total soluble proteins (B) in leaves of tomato plant infected with R. solani. Different letters denote significant changes among treatments within the same column, using the least significant difference test (p≤0.05).*

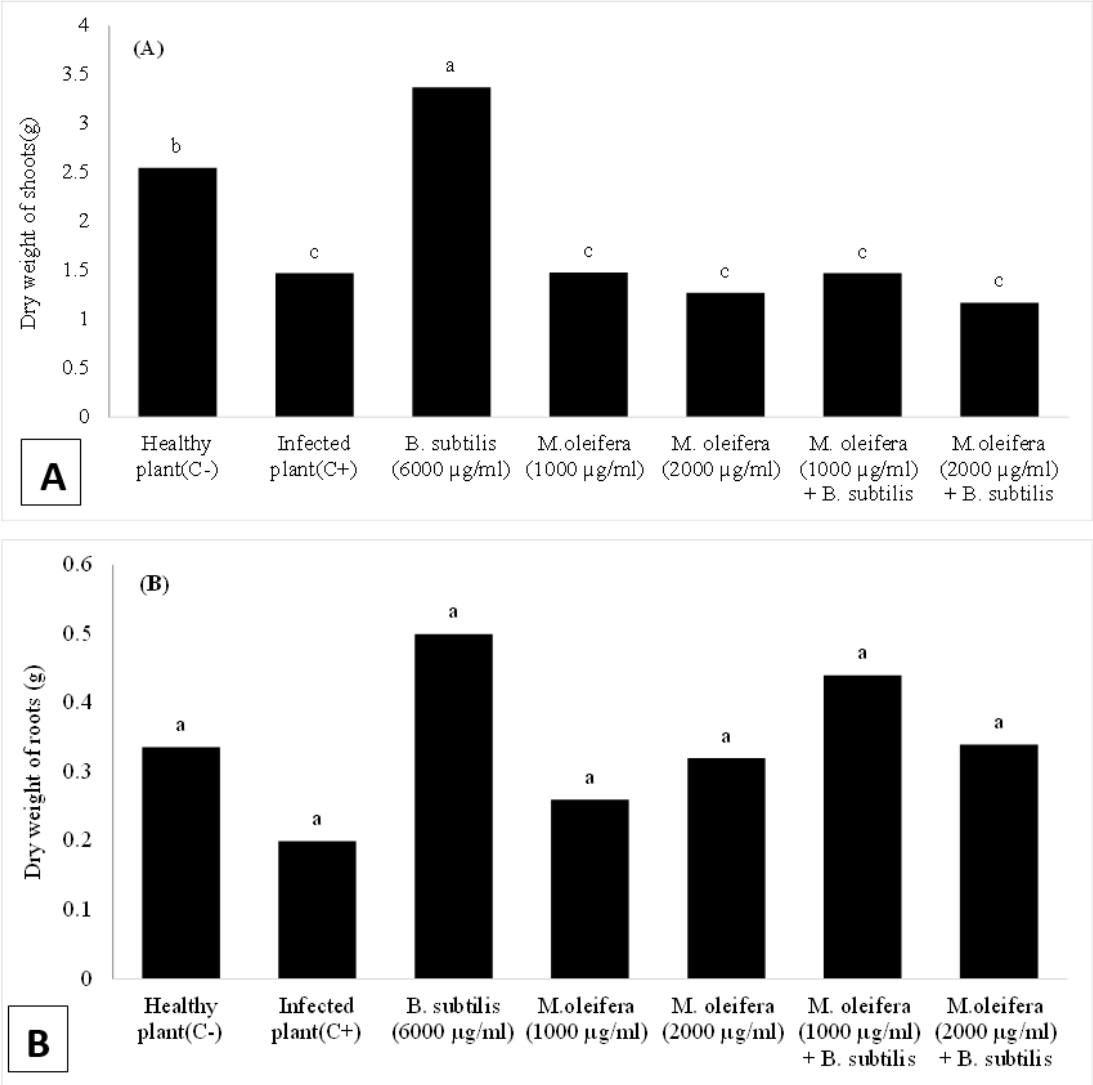


Figure 7: *Effect of M. oleifera extract, B. subtilis, and a combination of both on dray weight of shoot (A) and dray weight of roots (B) of tomato seedlings plants infected with R. solani. No significant differences were observed among treatments using the least significant difference test at (p≤0.05).*

The dry weight of roots expressed no appreciable differences among all the tested treatments, as the recorded values belonged to the same statistical group according to the LSD test at $p \leq 0.05$. Dry weight of roots values ranged from 0.20 g in infected plants to 0.50 g in plants treated with *B. subtilis* sting. Healthy plants recorded 0.336 g, while treatments with *M. oleifera* extract (1000 and 2000 µg/ml) resulted in dry weight of roots of 0.26 and 0.32 g, respectively. Combined treatments of *M. oleifera* extract (1000 and 2000 µg/ml) with *B. subtilis* sting (6000 µg/ml) recorded values of 0.44 and 0.34 g, respectively.

Discussion

Plant-derived extracts such as *M. oleifera* are rich in bioactive compounds, including tannins, saponins, terpenoids, alkaloids, flavonoids (e.g., quercetin and kaempferol), and glycosides, providing antimicrobial, antioxidant, and enzyme-inducing activities (Emad-ElDin *et al.*, 2016; Ahmadu *et al.*, 2020).

In the current study, GC-MS analysis of the crude leaf methanol extract of *M. oleifera* revealed a diverse profile of 39 bioactive compounds, many of which have been previously reported in other plant species. Our finding is in agreement with the results reported by Ahmed *et al.* (2024), who observed that the major constituents of *M. oleifera* extract included hexadecanoic acid, hexadecanoic acid ethyl ester, and 9,12,15-octadecatrienoic acid ethyl ester, along with notable compounds such as 2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-6-chromanol and tetratetracontane. These compounds were classified into several chemical groups, with fatty acids and fatty acid esters representing the predominant classes, fol-

lowed by steroids and sterols, terpenes, fatty alcohols, isoprenoids, and diterpene alcohols. Fatty acids and their derivatives have been widely reported to exhibit antifungal activity against plant pathogenic fungi by disrupting cell membrane integrity, inhibiting mycelial growth, and reducing pathogen biomass. For example, linolenic and linoleic acids substantially reduced mycelial growth of *R. solani in vitro*, indicating the effectiveness of fatty acid compounds against this pathogen (Walters *et al*., 2004). In contrast, sterols and chromanols are primarily associated with antioxidant protection, stabilizing cell membranes and scavenging free radicals, which may enhance plant defense responses under stress (Ahmed *et al*., 2024).

Bacillus spp. were well-recognized microbial biocontrol agents that offer a safe and sustainable strategy for managing plant pathogens such as *R. solani*. Such bacteria produce resilient endospores, enabling survival under harsh environmental conditions, and promote plant growth by inducing systemic resistance. They also act as antagonists through the secretion of diverse antimicrobial compounds, including lipopeptides, antibiotics, enzymes, and volatile organic compounds, while competing with pathogens for space and nutrients. Several *Bacillus*-based biopesticides have already been commercialized for the control of *R. solani*, demonstrating their practical potential in sustainable agriculture (Abbasa *et al*., 2019). Combination of these bioactive products may explain the synergistic effects observed in this plant methanol extract when applied against *R. solani* and support the potential use of *M. oleifera* as a natural biocontrol agent.

In *in vitro* assay against *R. solani*, the current investigation showed that *M. oleifera* methanol extract had antifungal activity. The combination of *M. oleifera* extract and *B. subtilis* sting displayed the best fungicidal activity against *R. solani*. Our *in vitro* results are consistent with Ahmed *et al*. (2024) who assessed the methanol crude extract of *M. oleifera* suppressing the radial growth of *Botrytis cinerea* (Pers,1794) demonstrating its antifungal effectiveness.Moreover, the current results are compatible with Majumder *et al*. (2024) who reported appreciable antifungal activity of *M. oleifera* leaves, which were extracted with different solvents (*i.e*., water, acetone, ethyl acetate, and hexane), and were tested against major soil-borne pathogens, including *R. solani* and *Fusarium* spp.

The current study's findings are consistent with a recent study conducted by Abdel-Hafez *et al*. (2021), which demonstrated that the *M. oleifera* extract had the highest impact on the linear growth of several fungal pathogens, including, *F. solani* (Mart.) (Sacc,1881), *F. oxysporium* (Schltdl, 1824), and *R. solani* upon using ethanol and aqueous extracts.

Our results are harmony with Al-Husnan *et al*. (2016) who reported that the aqueous extract of *M. olifera* leaves inhibited a broad range of fungi, including *Aspergillus niger* (van Tieghem, 1867), *A. flavus* (Link, 1809), *Alternaria* spp., *Fusarium* spp., *Rhizopus stolonifer* (Ehrenb.) (Vuillemin, 1902), and *Penicillium* sp., Additionally, *M. olifera* leaf ethanolic extract has shown antifungal efficacy against many dermatophytes (Chuang *et al*., 2007). Moreover, the obtained results are in agreement with the previous study reported by El-Mohamedy and Abdalla (2014), who assessed the efficacy of *M. oleifera* oil concentrations and discovered that the tested extracts inhibited the germination of spores *of F. oxysporum*, *Alternaria solani* (Sorauer, 1896), *A. alternate* (Fr.) (Keissl., 1912), and inhibited the germination of *R. solani, Sclerotium rolfsii* (Sacc,1911), and *Macrophomina phaseolina* (Tassi.) (Goid, 1947) with varying values.

Using a SEM, we observed reduction of the hyphal width of *R. solani* and loss of their structural integrity as a result of *M. oleifera* extract treatment. Additionally, shriveling, aberrant coiling, and lysis of hyphal filaments were observed, these results were referred to presence of a wide range of antimicrobial bioactive compounds in the *M. oleifera* extract able to inhibit the growth of the tested pathogen (Goss *et al*., 2017). *M. oleifera* leaves contain some crystalline alkaloids, fatty acid, proteins, glycosides, and niazirin suggested to be responsible for antimicrobial activities (Adandonon *et al*., 2006). However, there are few reports on the use of *M. oleifera* to control plant pathogens. Stoll (1988) reported the fungicidal effect of *M. oleifera* leaf extract on some soilborne fungi such as *Rhizoctonia* spp. The findings of this study confirmed that *M. oleifera* extract can be used as natural fungicides to control pathogenic fungi, reducing the dependence on the synthetic fungicides (El-Mohamedy *et al*., 2014). One of the most interesting results obtained in this study was using the effective biocontrol activity of the commercial formulation of *B. subtilis* (sting), which caused shrinkage, coiling, hyphal lysis, and breakage of the fungal mycelium. The reason was that the an-

tifungal compounds in culture supernatant produced by the *B. subtilis* sting inhibited mycelial growth of *R. solani* or killed the pathogenic fungi (Chen et al., 2016). Morphological changes of *R. solani* mycelium caused by *B. subtilis* sting were consistent with the conclusions of Ben *et al.* (2015) and Chen *et al.* (2016), which also revealed that the antifungal compounds present in *B. subtilis* filtrates play a key role in their biological control mechanisms. Rashad *et al.* (2022) showed that *B. subtilis* inhibited the mycelial growth of *R. solani*, indicating its potent antagonistic behavior. Several *Bacillus* spp. have been reported as biological control agents for plant diseases (Mnif *et al.*, 2015; Ali *et al.*, 2016; Gu *et al.*, 2017). They have convincing antagonistic properties because these bacteria synthesize broad-spectrum antibacterial compounds (Huang *et al.*, 2014). Antimicrobial compounds from *Bacillus* spp. protect the plants by directly inhibiting the invading pathogens.

In addition to the direct individual antifungal activity of *M. oleifera* and *B. subtilis* sting against *R. solani*, this study showed that using a combination of *M. oleifera* extract and *B. subtilis* sting against *R. solani* caused the pathogen hyphae to shrive, coil abnormally, break down and fragment, and ultimately lyse. These ultrastructural studies revealed the interactions between *R. solani* and the combination of *M. oleifera* extract and *B. subtilis* (sting), and attributed these effects to two types of antagonisms: hyperparasitism and antibiosis (Kumar *et al.*, 2013). The harmful effects of *M. oleifera* extract on the pathogenic fungi were restricted in: (a) partial or complete inhibition of mycelia growth, and (b) alternation in physiology and biochemistry of the fungal cells (Lee *et al.*, 2007; Chuang *et al.*, 2007; Hadi and Kashefi, 2013).

Under controlled greenhouse conditions, the present study demonstrated that application of *M. oleifera* extract at a concentration of 2000 µg/ml resulted in a relative disease control (RDC) of 62.79% against *R. solani*. Notably, the integrated treatment combining *M. oleifera* extract with *B. subtilis* sting at the recommended dose of 6000 µg/ml exhibited superior efficacy, achieving the highest reduction in root rot severity with an RDC of 88%.

These outcomes are consistent with the findings revealed by Goss *et al.* (2017), who reported that both leaf and seed extracts of *M. oleifera* substantially inhibited the mycelial growth of *R. solani* in cabbage. Their study further indicated that the two extract's types did not differ considerably in their antifungal performance and were effective in suppressing both *R. solani* and *F. solani*. In a previous study conducted by (Akinyeye *et al.*, 2014), the suppressive activity observed during weeks eight and nine after crop emergence was likely attributable to the abundance of flavonoids and phenolic constituents within *M. oleifera* extracts, where these compounds were being well-recognized by their broad-spectrum antimicrobial potential. Additional a study has similarly documented the capacity of *M. oleifera* extracts to disrupt essential metabolic pathways in the fungal pathogens, enhancing their disease-control effectiveness (Park *et al.*, 2011).

Our greenhouse results agree with Sehsah *et al.* (2022) who cleared that *B. subtilis* sting exhibited a pronounced capability to suppress *Cercospora* leaf spot severity in field evaluation. This reduction in disease intensity may be attributed to the rapid colonization of leaf surfaces by *B. subtilis* cells, creating a protective biological film that prevents pathogen spores from accessing natural openings and initiating infection. In addition, this bacterium's competitive ability for oxygen and nutrients on the phylloplane effectively deprived the pathogen from essential resources, limiting its growth and infection potential.

Biochemical analyses further indicated that all applied treatments appreciably enhanced the activities of PPO and POD. These enzymes play crucial roles in plant defense by oxidizing polyphenols into antimicrobial quinones and promoting the lignification of plant cell walls during pathogens invasion (Hassan *et al.*, 2007; She-ze *et al.*, 2008; Khalil *et al.*, 2022). Our obtained findings are in agreement with Sehsah *et al.* (2022), who reported that *M. oleifera* seed extract and *B. subtilis* cell suspension markedly increased PPO and POD activities in plants infected with *Cercospora* leaf spot.

The accumulation of phenolic compounds also played an essential role in reinforcing plant resistance. In this study, free phenols; known for their antimicrobial properties, were notably elevated in the treated plants. Phenolic compounds not only directly inhibit pathogenic microorganisms but also participate in enhancing the signaling pathways and act as precursors for lignin biosynthesis, providing additional structural resistance (Hammerschmidt, 2005). Currently, the

observed rise in total phenolic content was positively correlated with an enhanced plant disease resistance (Abo-Elyousr *et al*., 2009).

Moreover, the current treatments resulted in a marked increase in total soluble proteins content, important components of plant defense responses. These proteins accumulate rapidly during pathogen attack and contribute remarkably to suppressing disease development (Wang *et al*., 2005; Ahmed *et al*., 2022). The induction of genes encoding pathogenesis-related proteins (PRPs) is recognized as a critical mechanism for establishing durable and broad-spectrum resistance against fungal pathogens (Veronese *et al*., 1999).

Our results of plant defense responses are in agreement with Sehsah *et al*. (2022) who showed that the biochemical responses observed upon applying the *B. subtilis* and *M oleifera* extracts have activated multiple defense pathways, ultimately leading to remarkable suppression of *Cercospora* leaf spot.

Treatment of tomato plants with *B. subtilis* (sting) effectively reduced root rot disease caused by *R. solani,* leading to an increase in plant dry weight. Moreover, the application of *B. subtilis* sting appeared to stimulate overall plant growth, as evidenced by increases in both shoot and root dry weights. These findings are consistent with (Abbasa *et al*., 2019), who reported that *Bacillus* spp. play a substantial role in controlling diseases caused by *R. solani* and function as both biological control agents and plant growth promoters. Similarly, Rashad *et al*. (2022) observed that *B. subtilis* SR22 reduced *Rhizoctonia* root rot in tomato by up to 51% under greenhouse conditions, and enhanced most evaluated growth parameters by approximately 35%.

Earlier studies also support the current observations. SchmiedeKnecht *et al*. (1995) reported that bacterized tomato plants exhibited better growth, greener foliage, and higher yields compared to the untreated controls. Likewise, Bochow (1992) demonstrated that root treatment with *B. subtilis* increased rooting and mitigated disease symptoms following inoculation with *F. oxysporum f. sp. radicis-lycopersici*. Furthermore, the present results align with the previous findings reported by Gwa and Ige (2025), who revealed that *M. oleifera* extract displayed antifungal activity against *A. niger* in tomato plants. They added that increasing the concentration of *M. oleifera* extract was associated with enhanced plant height, leaf number, branch number, and leaf area even in the presence of higher *A. niger* spore concentrations.

Conclusions and Recommendations

Rhizoctonia root rot is a major threat to tomato production. This study demonstrated that *M. oleifera* leaf extract, *B. subtilis* sting, and their combination effectively suppressed *R. solani*. Leaf extract of *M. oleifera* was analyzed biochemically using Gas chromatography-mass spectrometry (GC-MS). In *in vitro* assay, *M. oleifera* extract achieved 85% inhibition against *R. solani* at 3000 µg/ml. The interactions among *M. oleifera* extract, *B. subtilis* sting, their combination, and *R. solani* were evaluated through scanning electron microscopy (SEM). Ultrastructural effects were studied and the results indicated that *M. oleifera* extract caused deformities with shriveling and abnormal coiling of pathogen's hyphal filaments. *B. subtilis* sting caused shrinkage, coiling, hyphal lysis, and breakage, while their combination caused hyphal breakdown, fragmentation, and hyphal lysis. *M. oleifera* extract at 2000 µg/ml reduced disease severity by 62.79%, while *B. subtilis* sting at 6000 µg/ml achieved 69.2% inhibition. The highest efficacy was observed with the combined treatment, achieving 88% relative disease control. The combined treatment also enhanced plant growth and defense plant responses, increasing PPO activity by 67.15%, POD by 78.18%, total phenols to 5404.12 µg/g fresh leaves, and total soluble proteins to 280.07 mg/g fresh leaves. Our findings indicate that integrating *B. subtilis* sting with *M. oleifera* extract represents a promising sustainable strategy for managing *Rhizoctonia* root rot, providing both effective disease suppression and growth promotion in tomato plants. This study recommends the use of the combined treatment of *M. oleifera* methanol extract and *B. subtilis* sting as a potential ecofriendly alternative to chemical fungicides, suitable for both laboratory and field applications, and highlights the importance of the bio-agents and biofungicides for disease management.

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oleifera leaf extract used in this study.

Novelty Statement

The novelty of the study lies in controlling *Rhizoctonia solani* root rot of tomato by using *Moringa oleifera* leaves extract, *Bacillus subtilis* (sting), and their combined application on tomato plants as environmentally safe and sustainable alternatives to chemical pesticides.

Author's Contribution

Rasha E. Selim: Conceptualization**,** methodology, investigations, data curation, writing review, editing and project administration.
Sahar M. Moussa: Methodology, data statistical analysis, writing review and editing.
Hanan F.B. Youssef: Conceptualization**,** methodology, investigations, data curation, writing review, editing and project administration.
The final version of the manuscript was approved by all the authors.

Ethical approval
Non-applicable.

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Generative AI and AI assisted technology statement
The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interests
The authors have declared no conflicts of interest.

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