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Gas chromatography-mass spectrometry comparative analysis of secondary metabolites produced by some *Bacillus* spp. as biocontrol agents against phytopathogenic fungi

Abdulaziz A. Al-Askar[a,*], Fatimah O. Al-Otibi[a], Gaber A. Abo-Zaid[b] , Ahmed Abdelkhalek[c]

[a] *Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia; aalaskara@ksu.edu.sa; falotibi@ksu.edu.sa*

[b] *Bioprocess Development Department, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications, Alexandria, 21934, Egypt; gaberam57@yahoo.com*

[c] *Plant Protection and Biomolecular Diagnosis Department, Arid Land Cultivation Research Institute, City of Scientific Research and Technological Applications, Alexandria 21934, Egypt; abdelkhalek2@yahoo.com*

Abstract

The widespread use of chemical fungicides is a current health and environmental concern. Hence, the quest for novel eco-friendly biocontrol agents poses a forthcoming challenge. In this study, 21 rhizosphere *Bacillus* isolates were isolated and assessed for their ability to inhibit *Rhizoctonia solani* growth. The four isolates (KSAR3, KSAR7, KSAR10, and KSAR11) demonstrated antagonistic activity, with the KSAR7 isolate displaying the greatest growth inhibition (55%). By sequencing the 16S rRNA gene, the four isolates were found to be *B. subtilis* (KSAR3 and KSAR10), *B. licheniformis* (KSAR11), and *B. tequilensis* (KSAR7). Using gas chromatography-mass spectrometry analysis, the chemical characterization and identification of the generated bacterial bioactive secondary metabolite were carried out. A total of 29 biologically active aliphatic and aromatic compounds were identified, among which bis(2-ethylhexyl) phthalate, dotriacontane, docosane, hexadecanoic acid, and octadecanoic acid were produced by the four *Bacillus* isolates. Additionally, seven distinct chemicals, including benzene, 1,3,5-trimethyl, were exclusively produced by the KSAR7 isolate. This may elucidate why it is more efficacious in suppressing *R. solani*. Ultimately, the results showed that *B. tequilensis* could be a good source of natural biocontrol antifungal agents for agriculture and food security, providing an alternative to synthetic pesticides.

Keywords*: Rhizoctonia solani; Bacillus tequilensis;* secondary metabolites; antifungal; GC-MS

1. Introduction

Plant pathogens result in substantial annual losses in agricultural yields. *Fusarium*, *Botrytis*, *Pythium*, *Sclerotinia*, *Verticillium*, and *Rhizoctonia* are examples of phytopathogenic fungi that can significantly reduce agricultural production [1]. *R. solani*, a pathogenic fungus, has the ability to infect approximately 250 plant species causing various diseases such as root rot disease [2]. These species belong to several families, such as *Amaranthaceae*, *Araceae*, *Asteraceae*, *Brassicaceae*, *Fabaceae*, *Linaceae Malvaceae*, *Moraceae*, *Poaceae*, *Rubiaceae*, and *Solanaceae* [3]. The management of *R. solani* has encountered numerous difficulties due to the absence of effective control measures using synthetic, semisynthetic, or biological agents [4]. Nevertheless, certain bacterial and fungal strains have been found as efficient bioremediation agents for reducing hazardous metal contamination in the environment [5–7]. For more than a century, the majority of farmers have depended on synthetic fungicides as the most effective option for controlling plant fungal diseases [8]. These have been damaged as a result of the ineffectiveness of fungicides and the adaptability mechanisms of plant diseases [9]. Furthermore, the impact of synthetic products on environmental pollution leads to the deposition of toxic residues on plants, increasing the diversity of robust pathogen strains [10–12]. This has sparked the need for alternative, environmentally sustainable methods of controlling plant diseases.

*Corresponding author e-mail: aalaskara@ksu.edu.sa

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Consequently, there has been a push to promote the adoption of integrated pest management by utilizing biological control as an alternative approach to pest management.

The utilization of biological control agents has led to a notable reduction in both the severity and incidence of plant diseases [13]. Microorganisms are used in agriculture to protect plants against different pathogens. The symbiotic relationship between plants and microorganisms is acknowledged for promoting plant growth by suppressing the growth of harmful fungi that affect plants. For more than 70 years, researchers have investigated the use of certain bacteria as biological control agents to combat soil-borne phytopathogens. This approach serves as an alternative or supplementary method for chemical disease control [14]. Biological control has minimal or insignificant adverse effects, guaranteeing effective and cost-effective long-term management. They have the ability to substitute for or greatly diminish reliance on chemical pesticides, thereby preventing not only immediate symptoms of poisoning but also subsequent disease. This strategy is consistent with ideas for integrated disease management [15]. Isolating new antagonistic bacteria is critical for the development of biocontrol techniques that target plant disease suppression. Several types of *Bacillus* have been shown to stop the growth of several phytopathogens, which makes them useful as biological control agents [16].

Bacillus is a strong microbe that fights against other microbes. It is considered a powerful biological control agent because it can stop pathogens from infecting plants [15]. The *Bacillus* genus employs a variety of strategies for biological regulation, including the synthesis of secondary metabolites such as siderophores, antibiotics, volatile extracellular metabolites, hydrolytic enzymes, hydrogen cyanide, and nutrient competition [1]. Furthermore, this includes activating systemic defense mechanisms and stimulating plant development [17,18]. The use of uncommon varieties in commercial biocontrol products may not produce desirable outcomes in indigenous soils due to variations in environmental variables. Indigenous bacteria, which are more well-suited, have the potential to surpass them in the process of establishing themselves in plants, impacting the effectiveness of plant growth-promoting rhizobacteria (PGPR). Hence, it is imperative to isolate and assess native strains as biological control agents [19]. Therefore, the current work sought to isolate and identify potential biological control agents by examining the in vitro antagonistic effects of *Bacillus* isolates against *Rhizoctonia solani*. Gas chromatography/mass spectrometry (GC-MS) was used to identify the secondary metabolites of the effective strains after they were characterized at the molecular level.

2. Materials and Methods

2.1. Source of phytopathogenic fungus

The *R. solani* fungus strain Rs34 (Accession number MW664425) causing tomato root rot disease was previously isolated and characterized [9]. *R. solani* was inoculated and maintained on potato dextrose agar medium at 28 °C under darkness for 6 days and stored in the same medium at 4 °C until used.

2.2. Isolation of *Bacillus* spp. from tomato rhizosphere

The soil samples were collected from the rhizosphere of healthy tomato (*Solanum lycopersicum* L.) plants in open fields in the Kingdom of Saudi Arabia. The undamaged root systems were excavated, and the loose soil attached to the area surrounding the roots (the rhizosphere) was carefully removed by shaking. The soil samples were gathered in aseptic polythene bags and transferred to the microbiology laboratory for bacterial isolation. One gram of soil sample was combined with 10 mL of sterile distilled water and agitated to prepare soil suspension. Three different plat replicates were prepared by spreading 200 µL of each of the dilutions (10^{-5}, 10^{-6}, and 10^{-7}), which were heated at 80 °C for 10 min to exclude vegetative cells, onto nutrient agar (NA) medium supplemented with the fungicide fluconazole to inhibit the fungal growth [20]. Afterward, the plates were placed in an incubator with a temperature set at 28 ± 2 °C for a period of 24 to 48 hours. Using a single colony isolation technique, different colonies were separated and purified on NA plates. They were then stored in glycerol stock at -20 °C for later use [21].

2.3. Assessing the fungicidal properties of *Bacillus* isolates

2.3.1. Preliminary assay for determining antifungal activity

Discs of *R. solani*, a phytopathogenic fungus with a diameter of 5 mm, were put in the center of PDA plates after being cultured for 5–6 days. The petri plates were divided into four sections, with each section being inoculated with a distinct *Bacillus*

isolate. Subsequently, the plates were incubated at a temperature of 28 ± 2 °C for seven days. *Bacillus* isolates that produced inhibitory zones against *R. solani* were chosen for future study [1].

2.3.2. Evaluation of the fungal growth inhibition percentage

The isolates that exhibited inhibitory zones against *R. solani* in the preliminary assay were chosen. The antagonistic impact and percentage growth inhibition were assessed using the dual culture technique [22,23]. Briefly, round fungal discs with a diameter of 5 mm were placed on the left side of the petri dish containing PDA media. At the same time, bacterial samples were introduced from the uppermost to the lowermost part of the right side of the plate. The petri plates were placed in an incubator set at a temperature of 28 ± 2 °C for seven days. The radius of the fungal colony that developed from the agar disc placed on the left side was utilized as a control for measurement. The percentage of inhibition in mycelium growth was determined using the formula: growth inhibition rate (%) = [(R−r)/R] × 100. In this context, R represents the radius measurement of the fungus colony on the control plate, while r represents the radius of fungal growth towards the bacterial treatment.

2.4. Molecular characterization of the selected bacterial isolates

The antagonistic bacterial isolates with antifungal activities that might be employed as biological control agents were molecularly identified by amplifying and sequencing the 16S ribosomal RNA (rRNA) gene [24]. Genomic DNA was isolated from 1 mL of an overnight bacterial culture using a bacterial genomic purification kit (Promega, USA) following the directions provided by the manufacturer. Subsequently, the genomic DNA was measured, and its ultimate concentration was modified to 100 ng of DNA per microliter. The 16S rRNA amplification was performed using the universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG. 3′) and 1492R (5′-CGGCTACCTTGTTACGACTT-3′). The PCR reaction mixture consisted of 12.5 µL of 2x PCR master mix, 2 µL of template DNA, 1 µL of each primer (10 pM), and 8.5 µL of nuclease-free water. The process of PCR amplification was carried out in a thermal cycler with the following specific conditions: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 52 °C for 30 seconds, and extension at 72 °C for 2 minutes. The last elongation stage is at 72 °C for 10 minutes. The amplified 16S rRNA was separated on an agarose gel by electrophoresis. The PCR results were subjected to direct sequencing following excision and purification from the gel using a PCR clean-up column kit. The sequencing was conducted using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and a model 3130xl Genetic Analyzer (Applied Biosystems, USA). The similarity percentage of the 16S rRNA sequences was assessed using the NCBI-BLAST tool available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The phylogenetic tree was generated using the MEGA 11 software tool, employing the UPGMA method and Bootstrap analysis with 2000 replications.

2.5. Identification of secondary metabolites using GC-MS

The bacterial culture filtrate was analyzed using GC–MS to determine the bioactive components responsible for the antifungal activity. Initially, a 48-hour bacterial culture broth was subjected to precipitation, and the resulting liquid above the sediment was gathered. This liquid was then combined with ethyl acetate, a solvent, in equal proportions of 1-part broth to 1-part ethyl acetate (volume/volume). The mixture was violently shaken for 20 minutes using an orbital shaker at a speed of 200 rpm. The ethyl acetate phase was isolated from the aqueous phase using a separating funnel. A rotary evaporator was used to concentrate the ethyl acetate phase at a temperature of 50 °C. A GC-MS system (TRACE 1300 Series, Thermo, USA) with a split-mode mass detector was used to look for secondary metabolite compounds in the concentrated ethyl acetate extract. Helium was used as the carrier gas at a flow rate of 1 mL/min. After 53 minutes of running at an energy level of 70 electron volts, the constituent mass spectra were determined by comparing them to data already in the GC-MS library [17,21,22].

2.6. Statistical analysis

The experiments were performed three times, and the data is presented as the mean value along with the standard deviation (mean ± SD). The data underwent analysis of variance (ANOVA), and Tukey's honest significant differences (H.S.D.) test was employed to assess disparities and establish the statistical significance of the process with a significance level of $p \leq 0.05$. Statistical significance was shown by letters arranged in decreasing order, with the relationship (a > b > c). The similar letters indicated no significant difference.

3. Results

3.1. Isolating, screening, and selecting antagonistic *Bacillus* strains against *Rhizoctonia solani*

A total of 21 bacterial isolates were obtained from the rhizosphere soil of tomato plants. Based on the first test to investigate its antifungal activity, only four isolates (19%), named KSAR3, KSAR7, KSAR10, and KSAR11, were able to antagonize *R. solani*. The remaining isolates (81%) didn't exhibit any activity against *R. solani*. In these cases, the fungus grew and covered the entire petri plate, identical to the control plates. Afterward, the antifungal efficacy of the four bioactive bacterial isolates was assessed as a percentage of growth inhibition. Figure 1 displays the antagonistic effects of these bacterial isolates using the dual culture technique. Figure 2 illustrates the growth inhibition percentage data displayed by different bacterial isolates. The maximum percent growth inhibition value was recorded at $54.9 \pm 0.68\%$ for isolate KSAR7, while KSAR3 exhibited the lowest inhibition percentage at $11.8 \pm 0.89\%$.

3.2. Molecular characterization of the selected bacterial isolates

During the process of isolating and purifying bacterial strains, only the strains that displayed the specific morphological features of *Bacillus* spp. were chosen. The colonies exhibited uneven and flattened boundaries, with a white, matte, or shiny tone and a floury, waxy, dry, or creamy appearance. The PCR reaction findings indicated that the amplicons of the 16S rRNA gene had a length of around 1500 base pairs. Based on the NCBI-BLAST alignment of 1450 bp of nucleotide sequence, the four isolates showed 96 to 99.8% homology to *Bacillus* spp. KSAR3 and KSAR10 had the highest similarity of 99.79% and 99.38%, respectively, with *B. subtilis* (Table 1). The two isolates were recorded as *B.* subtilis based on sequence homology results and phylogenetic tree analysis (Figure 3). They were given the accession numbers PQ212728 and PQ212730, respectively (Table 1). On the other hand, BLAST analysis of the nucleotide sequence of the KSAR11 isolate showed that it was very similar (99.72%) to other isolates of *B. licheniformis*. Under the accession number PQ212731, the annotated sequence was deposited in the GenBank database. Interestingly, the most potent bacterial isolate (KSAR7) that showed the highest antifungal activity showed the lowest percentage identity (96.03% of those reported in GenBank), suggesting it could be a new strain. The NCBI-BLAST alignment and phylogenetic tree analysis (Figure 3) showed that KSAR7 was very similar to the other *B. tequilensis* and came from the same lineage of evolution. It was deposited in the GenBank database under the name "*B. tequilensis* strain KSAR7" with the accession number PQ212729.

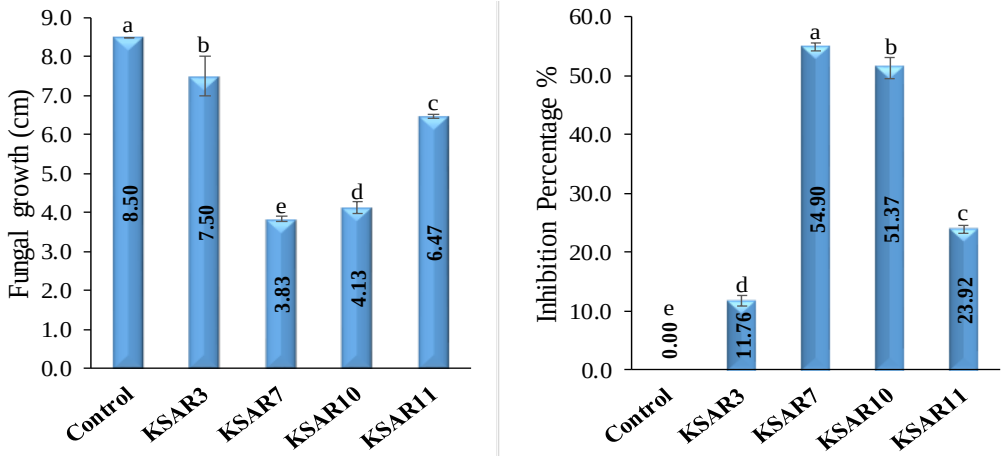

Figure 1. Effect of KSAR3, KSAR7, KSAR10, and KSAR11 isolates against *Rhizoctonia solani* growth compared with control under laboratory conditions.

Table 1. Molecularly identified *Bacillus* isolates: species name, sequence length, and GenBank accession number.

Key	Species name	Sequence length	Accession number	Closest strain in GenBank	Percentage identity
KSAR3	*Bacillus subtilis*	1453 bp	PQ212728	*B. subtilis*	99.79 %
KSAR7	*Bacillus tequilensis*	1459 bp	PQ212729	*B. tequilensis*	96.03 %
KSAR10	*Bacillus subtilis*	1458 bp	PQ212730	*B. subtilis*	99.38 %
KSAR11	*Bacillus licheniformis*	1446 bp	PQ212731	*B. licheniformis*	99.72 %

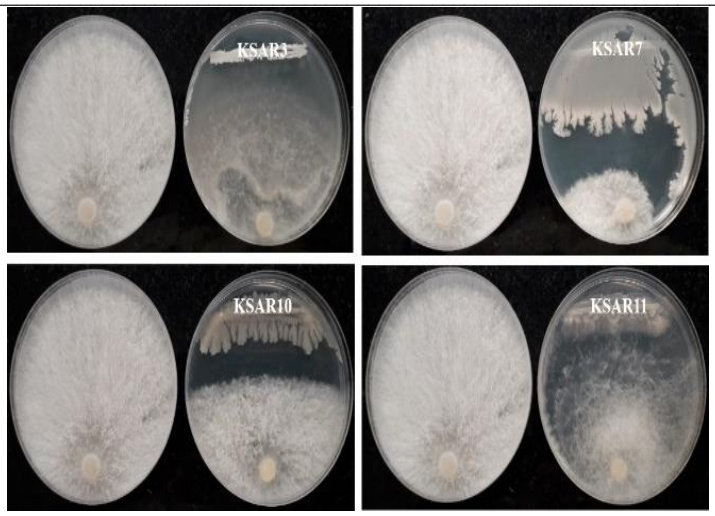

Figure 2. Antifungal activities of KSAR3, KSAR7, KSAR10, and KSAR11 *Bacillus* isolates against *Rhizoctonia solani*. Impact of isolates on fungal growth (left) and inhibition percentage of mycelium growth (right). The columns in the graph show the mean value of three replicated measurements, while the bars indicate the standard deviation. According to Tukey's HSD test with a significance threshold of 0.05, the values in each column assigned the same letter (a/b/c/d/e) do not exhibit any statistically significant differences.

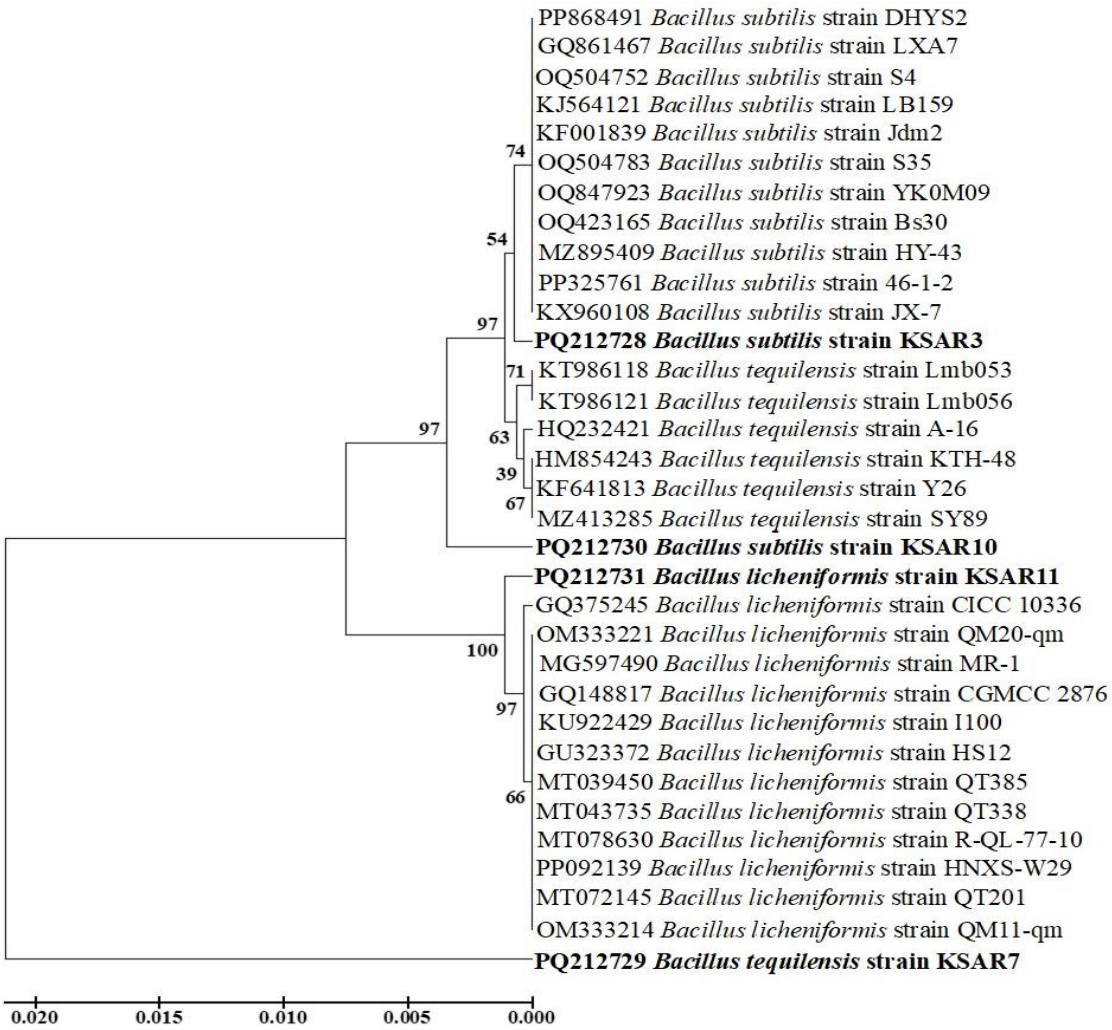

Figure 3. A phylogenetic tree shows how the isolated *Bacillus* strains (KSAR3, KSAR7, KSAR10, and KSAR11) are related to other isolates from GenBank. The tree is based on the nucleotide sequence of 16s rRNA gene. To create the tree, the MEGA 11 program used the UPGMA algorithm and the bootstrap method with 2000 repetitions.

3.3. Identification of secondary metabolites-produced *Bacillus* strains

The GC-MS analysis (Figure 4) showed that the ethyl acetate extract of the four bacterial strains contained 29 different compounds. These included several biologically active aliphatic and aromatic compounds. KSAR3 yielded a total of 15 compounds, with 8 being aliphatic and 7 being aromatic. On the other hand, KSAR7 produced a total of 24 compounds, consisting of 10 aliphatic and 14 aromatic compounds. KSAR10 yielded a total of 18 compounds, comprising 10 aliphatic compounds and 8 aromatic compounds. On the other hand, KSAR11 generated 10 compounds, consisting of 5 aliphatic compounds and 5 aromatic compounds (Figure 5).

The three aromatic compounds Bis(2-ethylhexyl) phthalate, Tris(2,4-di-tert-butylphenyl) phosphate, and Dibutyl phthalate showed the highest concentration (peak area %) among the other compounds (Figure 5). The bis(2-ethylhexyl) phthalate showed peak areas of 60.09, 30.14, 43.85, and 44.33 for KSAR3, KSAR7, KSAR10, and KSAR11, respectively. Tris(2,4-di-tert-butylphenyl) phosphate could not be found in the KSAR3 strain. On the other hand, dibutyl phthalate showed a peak area of 13.67, 7.13, 11.72, and 7.74 for KSAR3, KSAR7, KSAR10, and KSAR11. Dotriacontane, docosane, and hexadecanoic acid were the highest-produced aliphatic compounds, coming after aromatic compounds in order of concentration (Figure 5).

Other compounds, such as Hexadecane, Pentacosane, Docosane, 11-decyl-, Oxadecanoic acid, Tetradecane, 2,6,10-trimethyl, and Isochiapin B, were detected in lower concentrations (Figure 5). There were three compounds detected only in KSAR3: Hexadecanoic acid, ethyl ester, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, and i-Propyl-14-methylhexadecanoate. Their peak areas (%) were 0.57, 0.93, and 1.08, respectively (Figure 6). Similarly, glycan sialylated tetraose type 2, with a peak area of 2.64, was detected only in KSAR10. Interestingly, the KSAR7 strain that exhibited the highest antifungal activity produced an additional seven compounds that were not detected in any other bacterial strains (Figure 6). Six of these compounds were aromatic compounds. The highest compound was Benzene, 1,3,5-trimethyl (10.39%), while the lowest compound was Tetradecane (0.34%).

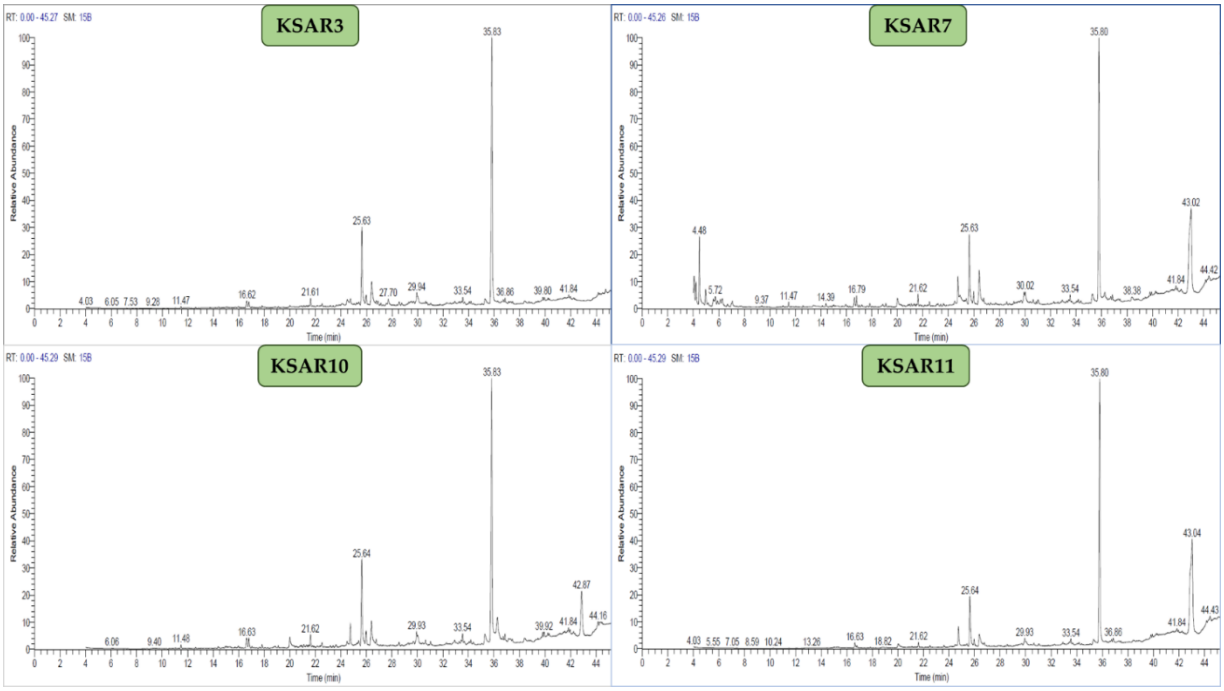

Figure 4. GC-MS fractionation of ethyl acetate extract of the culture filtrates of KSAR3, KSAR7, KSAR10, and KSAR11 strains.

4. Discussion

The overuse of synthetic chemicals for plant disease control objectives leads to environmental degradation, negative impacts on human health, and the emergence of disease resistance [25]. PGPR, particularly from the *Bacillus* genera, is gaining recognition for its widespread presence, ability to generate biological control agents and eco-friendly properties, making it a promising substitute for traditional pesticides and fertilizers [1,26,27]. In this

study, the antifungal properties of 21 different *Bacillus* isolates were assessed against *R. solani* using the dual culture technique. Out of these isolates, 4 (19%) demonstrated encouraging findings. The percentage growth inhibition results ranged from 11.8% to 55%. The KSAR7 demonstrated the highest level of activity, whereas the KSAR3 displayed the lowest level of antagonistic activity. Among the 1574 *Bacillus* isolates that were evaluated for their antifungal properties against *R. solani*, 118 isolates exhibited encouraging outcomes. The highest percentage of growth inhibition was observed at 55.6% against *R. solani* [1]. Lee et al. [28] evaluated 41 *Bacillus* isolates to determine their capacity to mitigate the effects of *Phytophthora capsici*. It was discovered that 12 of the isolates displayed antagonistic effects.

Recently, among 49 isolated bacteria, 10 *B. subtilis* isolates showed variable mycelial inhibition rates of *R. solani* in the co-culture technique, ranging from 7.54% to 38.10% [8]. Therefore, the outcome demonstrates that the KSAR7 isolate is highly valuable as a biological control agent against *R. solani* infection. The generation of antipathogenic secondary metabolites is the most well-known and crucial process by which antagonistic microorganisms inhibit plant pathogens [29]. So, in this study, we used GC-MS to look for biologically active compounds that might be linked to antifungal activity in the culture filtrate from the four bacterial isolates.

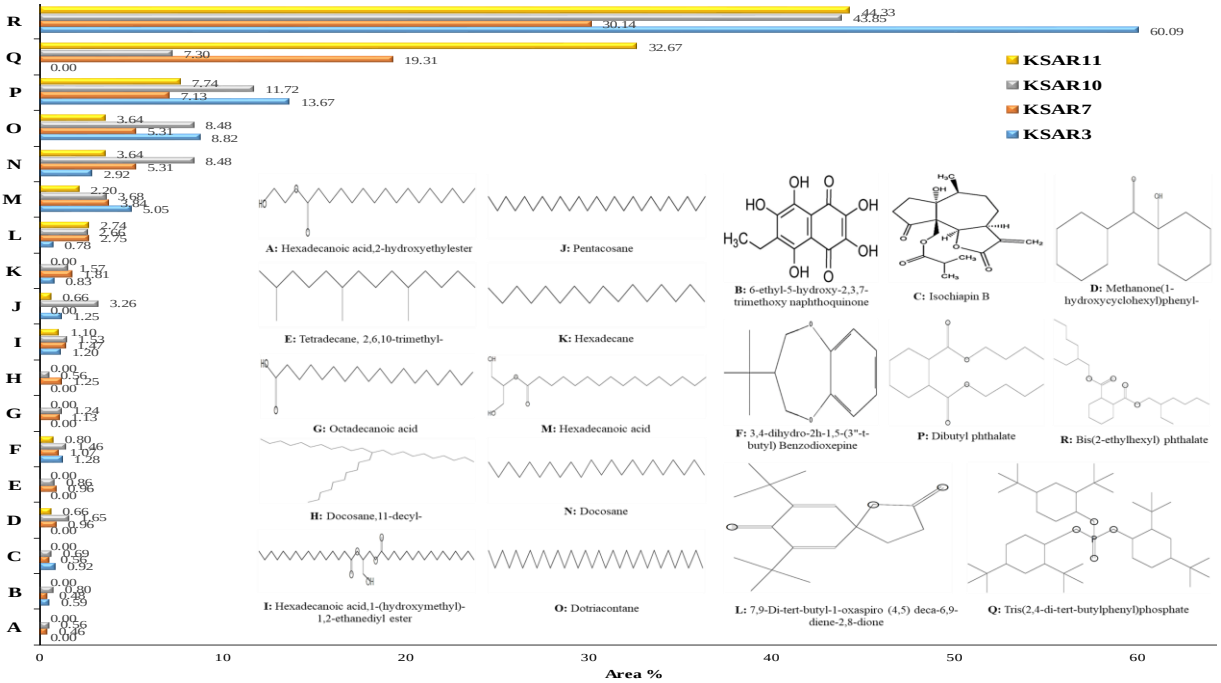

Figure 5. Comparison between the highest and common detected compounds in the ethyl acetate extract of the culture filtrates of KSAR3, KSAR7, KSAR10, and KSAR11 isolates.

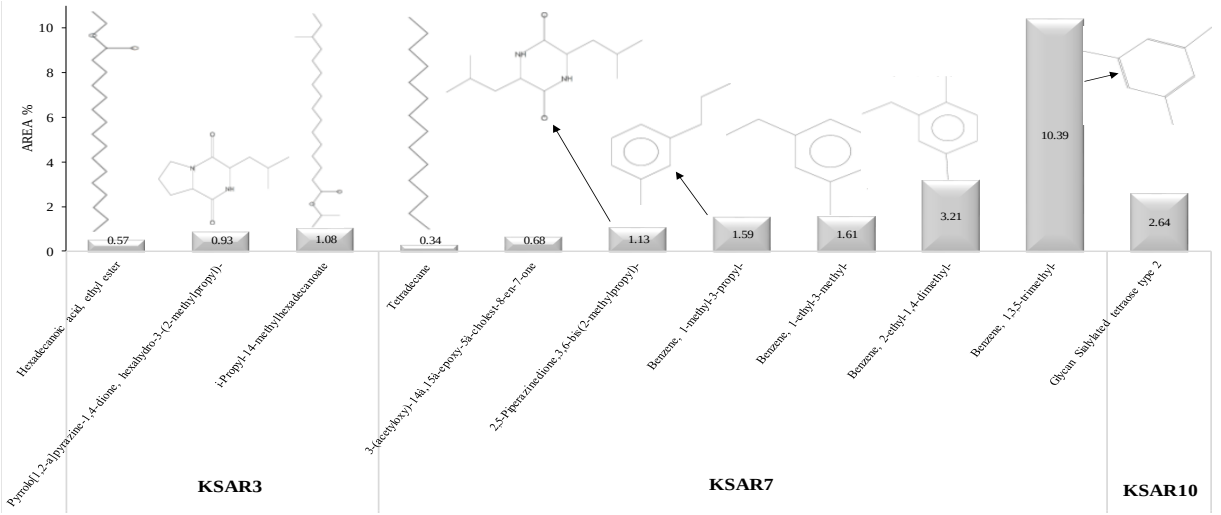

Figure 6. The unique detected compounds in the ethyl acetate extract of the culture filtrates of KSAR3, KSAR 7, and KSAR 10 isolates.

Bis(2-ethylhexyl) phthalate or Di(2-ethylhexyl) phthalate (DEHP) has been identified as a bioactive compound in a limited number of bacterial and fungal species [30]. The current study discovered that the four detected bacterial extracts contained DEHP and dibutyl phthalate, both of which have previously been reported to have antibacterial activity against unicellular and filamentous fungi [31]. Different biological activators for DEHP were reported. These included antimicrobial activities [32], antioxidant activity, antitumor activity, and antiviral [33,34], antibacterial, mosquito larvicidal, and cytotoxic properties [35]. The obtained results agree with Masrukhin et al. [36], who showed that DEHP was the primary component identified in the metabolites of *B. siamensis* that exhibited antifungal properties against *Cercospora lactucae*, *Colletotrichum gloeosporioides*, and *F. oxysporum*. Furthermore, the ability of *B. amyloliquefaciens* to inhibit the growth of *Alternaria alternative* is attributed to the synthesis of antifungal volatile compounds, specifically DEHP and dibutyl phthalate [37]. These compounds exhibit antibacterial and antifungal properties against both single-celled and filamentous fungi, as reported [31,38]. Multiple studies have demonstrated the antifungal properties of dibutyl phthalate [39,40], including its effectiveness against *R. solani* [41].

Dotriacontane, Docosane, and Hexadecanoic acid were the three most abundant aliphatic molecules synthesized by KSAR3, KSAR7, KSAR10, and KSAR11. Dotriacontane is a naturally occurring compound (alkanes) that can be found in soil compositions, where it is generated by plants or actinobacteria such as *Streptomyces capoamus* [42], *S. sparsus* [43], and *S. albus* [44]. Dotriacontane has a highly restricted range of documented antimicrobial activities. It exhibited potent antifungal activity against *A. tenuissima* and *A. alternate* [45]. As a result, further purification of this chemical and testing to establish its antifungal efficacy are required. Docosane is an organic fatty acid compound that is naturally volatile and has been found in many plant extracts [46–48], most recently in a culture extract of *Streptomyces* sp. MB 106 [45]. Its antibacterial properties have been documented recently [49]. Docosane displayed antimicrobial activities against *B. thuringiensis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* [50]. Hexadecanoic acid is a fatty acid that can be found in many medicinal plants, as well as in the byproducts of several *Bacillus* species. It possesses the potential to operate as an antifungal, antioxidant antibacterial, nematicide, anticancer, and pesticide compound [17,51–53]. It was shown that hexadecanoic acid extracts were able to inhibit the growth of *F. oxysporum* and *Xanthomonas campestris* [54]. Hexadecanoic acid exerts fungistatic effects by generating complex compounds when it binds to the active groups present on fungal cell walls [54]. Furthermore, the hydroxyl group of lipopolysaccharides, a component of the bacterial cell wall, reacts with hexadecanoic acid, altering the structure of the lipopolysaccharide membrane and causing it to become asymmetric. This leads to the lysis or destruction of the cell as a result of the disturbance in the equilibrium of the lipid membrane structure [55].

Among the 29 different compounds detected, 5 were produced only by KSAR7 and KSAR10 isolates. These compounds were Hexadecanoic acid, 2-hydroxyethyl ester, Methanone, (1-hydroxycyclohexyl)phenyl, Tetradecane, 2,6,10-trimethyl, Octadecanoic acid, and Docosane,11-decyl. Octadecanoic acid from the *B. atrophaeus* strain showed potential antifungal activity against *Verticillium* wilt [56]. Through in vitro antibacterial screening, it was shown that neem oil contains 9-octadecanoic acid and hexadecanoic acid, which effectively inhibit harmful bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* sp. [57]. Tetradecane, 2,6,10-trimethyl is well documented for its antioxidant, anti-inflammatory, antifungal, antibacterial, and nematicidal properties [58–60]. As a result, we think that the presence of these five compounds, along with other known compounds, helps explain why KSAR7 and KSAR10 are more effective at inhibiting *R. solani*. Furthermore, the discovery of seven unique compounds produced only by the KSAR7 isolate explains why it had the strongest antifungal effect against *R. solani*. Out of these seven compounds, four were aromatic compounds of benzene derivatives, with the highest one being Benzene, 1,3,5-trimethyl, with a peak area of 10.39%. It is a prominent volatile organic compound generated by *Pantoea* sp. Dez632 [60] and has demonstrated effective inhibition of plant pathogens [61].

The using of the 16S rRNA gene, a molecular marker for the identification of bacterial species, is a precise method for identifying bacterial species, while culture-based identification procedures can produce incorrect results [62,63]. The *Bacillus* strains identified in our study showed a sequence identity ranging from around 96% to 100%, with a sequence coverage of 99% to 100% in the sequence-similarity

comparison. The KSAR3 and KSAR10 isolates exhibited more than 99% similarity with *B. subtilis*. They were recorded in GenBank and identified as *B. subtilis* strain KSAR3, with accession number PQ212728, and *B. subtilis* strain KSAR10, with accession number PQ212730. KSAR11 shared more than 99% similarity with the *B. licheniformis* strains published in GenBank and was recorded as *B. licheniformis* strain KSAR11 with the accession number PQ212731. The genetic sequence KSAR7 exhibited a resemblance of 96% and lower with the *B. tequilensis* documented in GenBank. As a result, it was identified as *B. tequilensis* strain KSAR7 with accession number PQ212731. The KSAR7 sequence is less than 97% similar to the reported sequence in GenBank, indicating that this isolate could be recorded as a new strain [64,65]. However, to achieve this postulation, further deep molecular characterization is required.

5. Conclusions

The present study isolates and evaluates the effect of different *Bacillus* isolates on *R. solani*, the causal agent of root rot disease. Four isolates had antifungal activity. They were molecularly identified as four isolates: *B. subtilis* strain KSAR3, *B. tequilensis* strain KSAR7, *B. subtilis* strain KSAR10, and *B. licheniformis* strain KSAR11. The KSAR7 showed the highest inhibition growth (55%) against *R. solani*. The bioactive component profiles revealed that bis(2-ethylhexyl) phthalate, dotriacontane, docosane, hexadecanoic acid, and octadecanoic acid were the most common compounds found in four isolates. However, an additional seven distinct compounds were detected only in the KSAR7 metabolites. Such specific compounds require additional purification and assays to confirm their antifungal activity. Interestingly, the significance of *B. tequilensis* as an antifungal agent has rarely been studied. As a result, *B. tequilensis'* ability to suppress *R. solani* growth validates its potential use as a biocontrol agent for root rot disease management.

Conflicts of interest

There are no conflicts to declare.

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