Biocontrol Potential of *Bacillus subtilis* Against Phytopathogenic Fungi and *Spodoptera* Species: Toxicological Evaluation and GC–MS Characterization

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Abstract: Phytopathogenic fungi and lepidopteran insect pests pose significant constraints to agricultural productivity worldwide, while the excessive use of chemical pesticides has led to resistance development, environmental contamination, and risks to human health. In the present study, the biocontrol potential of *Bacillus subtilis* was evaluated against major soil-borne fungal pathogens (*Fusarium solani*, *Fusarium oxysporum*, *Rhizoctonia solani*, and *Sclerotium rolfsii*) and the economically important insect pests *Spodoptera littoralis* and *Spodoptera frugiperda* under laboratory conditions. Antagonistic activity against phytopathogenic fungi was assessed using dual culture and poisoned food techniques, while larvicidal efficacy against first instar larvae of *Spodoptera* species was determined using time- and concentration-dependent bioassays. Results demonstrated that *B. subtilis* exhibited strong antagonistic activity against fungal pathogens, particularly *R. solani* and *F. oxysporum*, with higher inhibition observed when the bacterium was inoculated prior to pathogen introduction. The crude ethyl acetate extract significantly reduced fungal mycelial growth, with EC_{50} values of 924.6 and 1574.8 ppm against *R. solani* and *F. oxysporum*, respectively. In insect bioassays, *B. subtilis* culture showed higher larvicidal activity than its crude extract against both *S. littoralis* and *S. frugiperda*, with mortality increasing in a time-dependent manner and LC_{50} values decreasing with prolonged exposure. GC–MS analysis of the crude extract revealed a diverse profile of bioactive metabolites, including fatty acids, fatty alcohols, terpenoid-related compounds, and aromatic acid derivatives, which are known to exert antifungal and insecticidal effects through membrane disruption, metabolic interference, and hormonal dysregulation. The results highlight *B. subtilis* as a promising eco-friendly biocontrol agent with dual activity against phytopathogenic fungi and lepidopteran pests, supporting its potential application in integrated pest and disease management programs.

Keywords: *Bacillus subtilis*, biocontrol, phytopathogenic fungi, *Spodoptera littoralis*, *Spodoptera frugiperda*, GC–MS, larvicidal activity

1.Introduction:

Phytopathogenic fungi and insect pests are considered as a major threat to global agricultural productivity and food security. Soil-borne fungi, particularly *Fusarium* spp. and *Rhizoctonia solani*, are among the most destructive plant pathogens due to their persistence in the rhizosphere and their ability to survive for extended periods. *Fusarium oxysporum* causes vascular wilt and root rot by colonizing the xylem, disrupting water transport and leading to chlorosis, wilting, and plant death across a broad host range, including tomato, banana, and legumes, while also producing mycotoxins such as fumonisins that pose risks to human and animal health**.** In contrast, *R. solani* is a necrotrophic pathogen responsible for damping-off, brown patch, and wire stem diseases, primarily affecting seeds and seedlings and persisting in soil via resilient sclerotia. Concurrently, the cotton leaf-worm, *Spodoptera littoralis* and the fall armyworm, *Spodoptera frugiperda* are among the most destructive lepidopteran pests worldwide, causing severe yield losses in cotton, maize, vegetables, and other crops due to their polyphagy, high reproductive potential, and adaptability **(El-Defrawi *et al*., 1964; El-Helaly *et al*., 2020; Bakry and Abdel-Baky, 2023).** Although chemical fungicides and insecticides remain widely used, their intensive application has resulted in resistance development, environmental contamination, and adverse effects on non-target organisms and human health **(Li *et al*., 2007; Isman, 2006).** Consequently, biological control strategies have gained increasing attention as sustainable and environmentally benign alternatives **(Campbell, 1989)**. Among microbial biocontrol agents, *Bacillus subtilis*, a Gram-positive, spore-forming bacterium, has emerged as a promising bio-fungicide and bio-insecticide due to its ecological fitness and capacity to produce a diverse array of bioactive secondary metabolites, including lipopeptides (surfactins, iturins, and fengycins), fatty acids, esters, sulfur-containing compounds, and nitrogenous derivatives **(Desbois & Smith, 2010; Ahmad *et al*., 2013)**. These metabolites suppress phytopathogenic fungi through membrane disruption,

competitive root colonization, biofilm formation, and induction of systemic resistance in plants, while also exerting insecticidal effects via midgut epithelial damage, inhibition of digestive and detoxification enzymes, neurotoxic interference, and behavioral deterrence **(Bloomquist, 2009; Senthil-Nathan, 2013; Qasim *et al.*, 2024)**. Despite the extensive commercialization of *Bacillus thuringiensis* for insect control **(Koul *et al.*, 2004)**, comparative studies assessing the larvicidal efficacy, time-dependent toxicity, and chemical basis of *B. subtilis* metabolites against early larval instars of *S. littoralis* and *S. frugiperda* remain limited. Therefore, the present study aimed to evaluate the larvicidal activity of *Bacillus subtilis* culture and its extract against first instar larvae of *S. littoralis* and *S. frugiperda* under laboratory conditions and to identify the bioactive metabolites responsible for toxicity using GC–MS analysis.

2.Materials and Methods:

2.1.Bacterial strain

The bacterial isolate *Bacillus subtilis* used in the present study was previously isolated and further identified using the 16S rRNA method (**Mohamed, 2020**).

2.2. Phytopathogenic fungi

2.2.1.Isolation and purification of phytopathogenic fungi

Naturally-diseased tomato plants showing root rot and wilt symptoms were collected from private fields in Bani-sueif Governorate, Egypt. Plant root and stem sample were thoroughly rinsed under running tap water to remove soil particles and surface-sterilized by immersion in 1% sodium hypochlorite (NaOCl) solution for 1minute followed by three rinses with sterile distilled water **(Burr *et al.*, 1978)** Small pieces of plant tissue (~3-5 mm) were cut aseptically and plated onto Potato Dextrose Agar (PDA) medium supplemented with 100 mg/L streptomycin sulfate to suppress bacterial growth. Plates were incubated at 27 °C for 5-7 days. Fungal colonies were subcultured onto fresh PDA plates to obtain pure cultures. Single-spore isolation and hyphal tip techniques **(Dhingra and Sinclair, 1984)** were performed to ensure culture purity.

2.2.2. Pathogen identification

Morphological characteristics of the purified fungal isolates were examined under a compound microscope, including conidial shape and size. The identification was verified in the Plant Pathology Department, Faculty of Agriculture, Cairo University. Isolates confirmed as *Rhizoctonia solani* and *Fusarium oxysporum*, *F. solani* and *Sclerotium rolfsii* were maintained on PDA slants at 4 °C.

2.3. Antagonistic activity of *B. subtilis* towards pathogenic fungi

The antagonistic efficacy of *B. subtilis* against *R. solani*, *F. oxysporum*, *F. solani*, and *S. rolfsii* was evaluated using the dual culture technique.

2.3.1.Dual Culture assay

Sterile Petri dishes (90 mm diameter) were poured with 20 ml of molten PDA under aseptic conditions in a laminar airflow chamber. After solidification, a 5 mm diameter mycelial disc of pathogenic fungi (7-day-old culture) was placed at one end of the plate (10 mm from the edge). On the opposite end of the same plate, *B. subtilis* was streaked at an equal distance from the edge. Plates inoculated with only the pathogen served as a control. All plates were incubated at 27 ± 2°C for 7 days. The zone of inhibition was recorded when the mycelium mats of pathogens cover the medium surface of the control plates. Two sets of experiments were executed, in the first, the PDA plates were seeded with both bioagents and pathogens at the same time. In the second, bioagents were introduced into the PDA plates 24 hr. prior to the pathogenic fungi **(Arzanlou *et al.*, 2016).**

2.4.Production and Extraction of *B. subtilis* crude extract

The bacterial strain *(B. subtilis*) was fermented in King's B medium (g/L): peptone 20 g, $MgSO_4$ 1.5 g, K_2HPO_4 1.5 g, Glycerol 10 ml, Agar 15 g, pH 7.0. for 120 hr. at 30º C. The bacterial culture was centrifuged for 10 min at 12500 rpm to remove bacterial cells. The supernatant was then extracted three times with ethyl acetate at the ratio of 1:1 (v/v) and shaken vigorously for 30 min. to obtain a crude extract, the organic layer was collected and evaporated to dryness in a vacuum evaporator at 40º C. The residues were dissolved in methanol and kept at 4ºC**. (Almalki., 2020)**

2.5. Antifungal activity of crude extract

The activity of the crude extract was determined by the poisoned food method. The residue of ethyl acetate was dissolved in dimethylsulphoxide (DMSO) to determine the antagonistic activity of crude extract at 2000, 1000, 500, 250 ppm, added to PDA media, poured in sterile petri dishes and allowed to solidify, PDA without crude extract was used as a control. Media were then surfacely seeded with 5 mm discs of 5-7 days old pathogenic fungal cultures. Petri dishes were incubated for 5-7 days at 25±2 ºC, and the percentage of growth inhibition was calculated using the.

following formula:

Growth inhibition% = R_1-R_2/R_1×100

where: R_1, the radius of normal growth in control; R_2, the radius of the inhibited growth in the treatment **(Paul, *et al.*, 2007).**

2.6. Insect bioassay

2.6.1.Insects rearing technique

2.6.2.The cotton leaf-worm *Spodoptera littoralis*

A stock culture of susceptible strain cotton leaf-worm, *Spodoptera littoralis* (Boisd) was obtained under precise laboratory conditions at the Bioassay Department, Central Pesticides Laboratory, Agricultural Research Center, Dokki, Giza, the strain was maintained for several generations without exposure to insecticidal and /or microbial pressure, ensuring genetic fidelity. The insect was reared on castor-oil leaves, *Ricinus communis*, under laboratory conditions at 25±2°C and 60±5% R.H. **(El-Defrawi *et al.*, 1964)**. First- instar larvae were systematically used for the present study to assess their biological responses.

2.6.3.The fall armyworm, *Spodoptera frugiperda*

The fall armyworm, *Spodoptera frugiperda* (Smith), was maintained under controlled laboratory conditions (28 ± 2°C, 60 ± 5% relative humidity, and a photoperiod of 14 hours' light: 10 hours' dark) for no less than 15 generations at the Bioassay Department of the Central Pesticides Laboratory, Agricultural Research Center, Dokki, Giza. During this period, the strain was kept free from chemical exposure or pesticide influence. Larvae were reared on fresh castor leaves (*Ricinus communis*) within large glass containers (2 L) until pupation occurred. Upon emergence as adults, moths were transferred to mating chambers—glass jars containing sugar-soaked cotton (10% solution) for nourishment, alongside castor leaves for egg deposition. Subsequently, the resulting egg clusters were housed in glass containers until hatching **(Kruger *et al.*, 2012**). The newly hatched first instar larvae of *S. frugiperda* were used in the present study.

2.7.Experimental techniques

2.7.1.The diet preparation

The semi-synthetic diet utilized in this study was prepared following the method described by **(Shorey and Halo, 1965)** to assess the effectiveness of *Bacillus subtilis* and its extract against the first larval instars of *Spodoptera littoralis* and *Spodoptera frugiperda*. The diet comprised kidney bean flour (150 g), yeast powder (15 g), ascorbic acid (3 g), methyl p-hydroxybenzoate (3 g), formaldehyde (40%) at 2 ml, agar (6 g), and 600 ml of sterilized distilled water.

To prepare the diet, all dry ingredients, except for the agar, were carefully weighed and placed in a clean, sterilized glass beaker containing 400 ml of sterilized distilled water. These were stirred with an electric mixer until fully homogeneous. The agar was dissolved in the remaining 200 ml of water, which was heated in a water bath at 100°C for 5 minutes. Once the agar solution cooled to approximately 60°C, it was combined with the blended ingredients. Formaldehyde was then added to the mixture and thoroughly mixed. Finally, the prepared diet was poured into a specified number of sterilized plastic Petri dishes (5 cm diameter × 2 cm height) and left to cool and solidify.

2.7.2.The *Bacillus subtilis* serial concentrations preparation

The *B. subtilis* and its crude extract activity were assessed on newly ecdysed first instar larvae of *S. littoralis* and *S. frugiperda*. Four different concentrations were prepared from a stock solution for *B. subtilis* culture ($32 X10^8$, $16 X10^8$, $8 X10^8$ and $4 X10^8$ ml) while extract concentrations were (50, 25,12.5,6.25%) by diluting in satirized distill water in a volumetric flask, achieving the necessary concentrations to induce 20-80 % mortality.

The insect's mortality was recorded at 1,2,3,5 and 7 days' post-exposure with accumulative larval mortality was assessed after 7 days from exposure. A contact thin film bioassay was performed by exposing the first instar larvae to an equal amount of artificial diet saturated with each concentration (500 µl) for each concentration and allowing it to dry at room temperature before feeding to the insects. Three replicates were conducted, each consisting of 10 adults for *S. littorals*, while *S. frugiperda* individuals were treated separately to minimize cannibalism. A control group was included, treated only with the distilled water. All bioassays were accomplished under the same laboratory conditions as previously described.

Mortality percentages were corrected by Abbott's formula **(Abbott,1925)**. Lethal concentration values (LC_{50}), confidence limits, and slopes were calculated using regression analysis with LDP line software **(Bakr, 2007).**

2.8. Gas chromatography–mass spectrometry (GC-MS) analysisIII

The chemical composition of the sample performed using GC-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). The column oven temperature was initially held at 60°C and then increased by 5°C /min to 250°C withhold 2 min then increased to 300 with 30 C/min. The injector temperature was kept at 270°C. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 4 min and diluted samples of 1 µl were injected automatically using Auto sampler AS3000 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 50–650 in full scan mode. The ion source and transfer line were set at 200 °C and 280 °C, respectively. The components were identified by comparison of their mass spectra with those of WILEY 09 and NIST14 mass spectral database.

3.RESULTS AND DISCUSSION:

3.1. Toxicological study of *Bacillus subtilis* culture and its crude extract against phytopathogenic fungi (*F.solani, S. rolfsii, R. solani* and *F. oxysporum*) under laboratory conditions

3.1.1.Antagonistic effect of *B. subtilis* against fungal pathogens

The antagonistic effect of *B. subtilis* against four pathogenic fungi *F. solani*, *S. rolfsii*. *solani* and *F. oxysporum* was evaluated using the dual culture technique (Table 1. and Fig 1.).

3.1.2. Dual culture assay

The dual culture assay revealed that *B. subtilis* had high antagonistic activity against fungal pathogens. The antagonistic effect was measured through the determination of the inhibition zone (mm). *B. subtilis* significantly inhibited the mycelium growth of *F.solani*, *S. rolfsii*, *R. solani* and *F. oxysporum*. In the case of inoculation with the bioagent 24 h before the pathogen, the effect of the bioagent increased and the inhibition zone increased with all phytopathogenic fungi. The highest inhibition zone was recorded with *R. solani* (23 mm) followed by *F. oxysporium* (19 mm), in the case of inoculation with the *B. subtilis* 24 h before the pathogens. On the other hand, the lowest inhibition zone was recorded with *F. solani* (12 mm) and *S. rolfsii* (14 mm) in the case of inoculation with the bioagent and the pathogen at the same time. *B. subtilis* showed a stronger suppression against *R. solani* and *F. oxysporum*, so these two fungal pathogen chosen for the following experiments.

Table 1. Antagonistic effect of *B. subtilis* against phytopathogenic fungi

Phytopathogenic fungi	Inhibition zone (mm)	
	24h before	At the same time
R. solani	23	18
F. solani	15	12
F. oxysporum	19	15
S. rolfsii	17	14

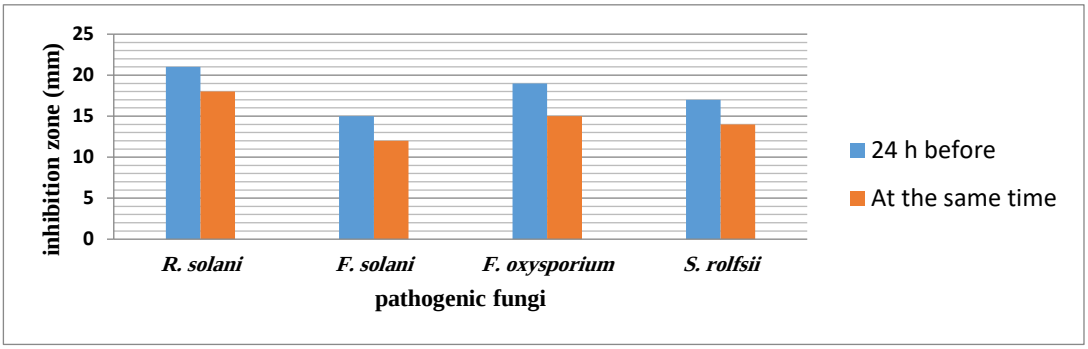

Fig.1.Antagonistic effect of *Bacillus subtilis* against phytopathogenic fungi

3.2. Antifungal activity of ethyl acetate crude extract

The crude extract of *B. subtilis* was tested for its antifungal activity against the phytopathogenic fungi at the concentrations of 2000, 1000, 500, and 250 ppm (Table 2). The crude extract recorded the maximum reduction (66 and 55.5%) of mycelial growth against *R. solani* and *F. oxysporum*, respectively, at the higher concentration. All tested concentrations reduced the mycelium growth of the tested fungi. *R. solani* and *F. oxysporum* recorded EC_{50} (924.6 and 1574.8), respectively; hence, we can conclude that the effect of crude extract was more effective on *R. solani* than *F. oxysporum*.

Table 2. Efficacy of crude extract produced by *B. subtilis* against phytopathogenic fungi.

Concentrations (ppm)	Reduction in mycelium growth	
	R. solani	***F. oxysporium***
2000	66	55.5
1000	52	40
500	37	33
250	24	22
control	0	0
EC_{50}	924.6	1574.8

3.3.Toxicological study of *Bacillus subtilis* culture and its crude extract against *Spodoptera littoralis* and *Spodoptera frugiperda* under laboratory conditions

The study was evaluated the efficacy of *Bacillus subtillis* bacterium in a culture form and its extract as a new biocontrol agent against two major agriculture pest (*S. littoralis* and *S. frugiperda*) to find alternative and supplements compounds to conventional chemical insecticides.

The results presented in Table (3) and depicted in Fig. (2), shows the susceptibility of new ecdysed of 1st larval instar *S. littoralis* and *S. frugiperda* to four concentrations of *B. Subtillis* culture as follows ($32x10^8$,$16x10^8$,$8x10^8$ and $4x10^8$ per ml) after exposure and feeding to contaminated kidney bean media over various time intervals1 ,2 ,3 ,5 and 7 days of treatment. It is clear from data the larva mortality increasing depending on time exposure. In general, all concentrations of *B. subtilis* culture against *S. littoralis* achieved high mortality. But the concentrations $32x10^8$ and $16 x10^8$ were the most effective concentration against 1st larval instar of *S. littoralis* it shows highly mortality by (66.7,76.7,96.7,96.7 and100 % then concentration $16 x10^8$ by mortality % (30,53.3,80,93.3 and100) after 1,2,3,5and 7 days from exposure, then concentration $8 x10^8$ with mortality % (23.3,46.7,53.3,76.7 and 80%) finally concentration $4 x10^8$ recorded mortality % by (3.3,26.7,40,43.3and 76.7%) after 1,2,3,5and 7 days, respectively. The candidate of LC_{50} values were arranged according to time of exposure in the following descending order (1.9 ,4.3,6.0,10.8 and 22.0 $x10^8$ /ml) after 7, 5, 3, 2 and 1 days from exposure, respectively. In the case of *S. frugiperda*, the highest mortality was recorded by concentration $32x10^8$, which recorded (56.7,73.3,100,100,100%), then concentration $16x10^8$ by recorded mortality % by about (50,66.7,83.3,96.7 and 100%), then concentration $8x10^8$ by (30,43.3,46.7,56.7 and 70%) after 1,2,3,5and 7 days from exposure, respectively. While concentration $4 x10^8$ had moderate mortality % by (20,23.3,23.3,36.7 and 46.7%) after 1,2,3,5and 7 days from exposure. The LC_{50} values for the candidate were organized in descending order based on the time of exposure: after 7, 5, 3, 2, and 1 day, the values were 4.4, 5.7, 7.6, 10.5, and 20.2 x 10^8 /ml, respectively.

On the other hand, the concentrations were used for *B. subtillis* extract were 50, 25,12.5 and 6.25%. The different concentrations 50, 25, 12.5% the data shows that the effectiveness increase over time for instance the mortality percentage recorded highly toxicity start from day 2nd to 7th day against 1st larval instar of *S. littoralis* the mortality rates ranged from 46.7 to 93.3% depending on concentration, while the lowest concentration 6.25% recorded moderate mortality from 3rd day to 7th day rate ranged from 34.4 to 68.8 %. on the other side the all concentration of *B. subtillis* crude extract against 1st larval instar of *S. frugiperda* were recorded high mortality at 7th day mortality rates ranged from 47.8 to 63.3% in addition moderate mortality from 2nd day to 5th day ranged from 20 to 50%. The LC_{50} values for the candidate were organized in descending order based on the time of exposure: after 7, 5, 3, 2, and 1 day, the values were 10.23, 51.38, 63.17, 93.05, and 140.38 %, respectively

According to the mortality percentage values observed in Table (3&4) concluded that *B. subtillis* culture was more effective than its crude extract against the first larval instar for both *S. littoralis* and *S. frugiperda* and, indicating that the duration of exposure has a significant effect on increasing the insect's mortality.

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Table 3. Time- and concentration-dependent larvicidal activity of *Bacillus subtilis* culture against first instar larvae of *Spodoptera littoralis* and *Spodoptera frugiperda* under laboratory conditions

***B.s* culture conc. (ml)**	***Spodoptera littoralis*** **Mortality % based on exposure time**					***Spodoptera frugiperda*** **Mortality % based on exposure time**				
	1st day	**2nd day**	**3rd day**	**5th day**	**7th day**	**1st day**	**2nd day**	**3rd day**	**5th day**	**7th day**
$32x10^8$	66.7	76.7	96.7	96.7	100	56.7	73.3	100	100	100
$16x10^8$	30	53.3	80	93.3	100	50	66.7	83.3	96.7	100
$8x10^8$	23.3	46.7	53.3	76.7	80	30	43.3	46.7	56.7	70
$4x10^8$	3.3	26.7	40	43.3	76.7	20	23.3	23.3	36.7	46.7
LC_{50}	22.0	10.8	6.0	4.3	1.9	20.2	10.5	7.6	5.7	4.4
Control	0	0	0	0	0	0	0	0	0	0

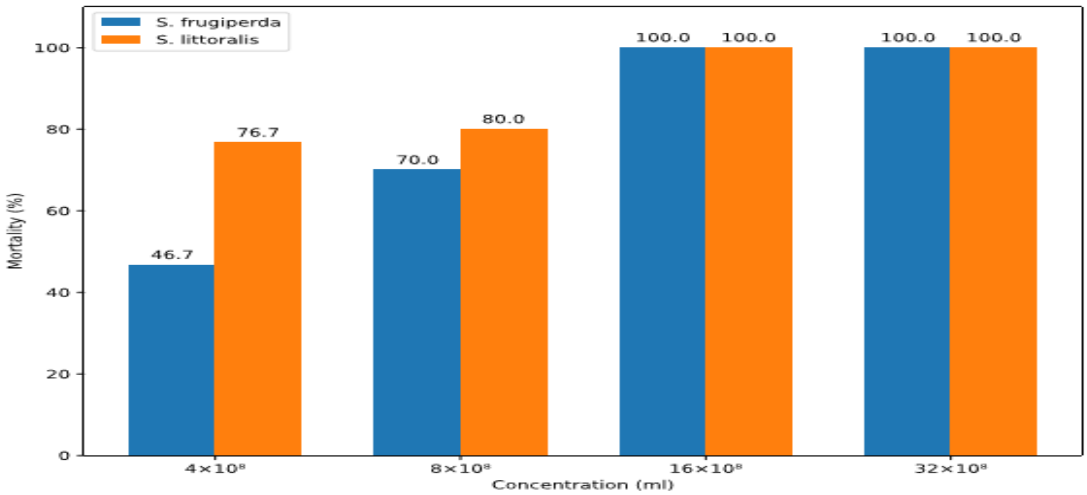

Fig. 2. Cumulative mortality percentage after 7 days of mortality response of the first instar larvae of *S. littoralis* and *S. frugiperda* to the *Bacillus subtilis* culture after7 days under laboratory conditions

Table 4. Time- and concentration-dependent larvicidal activity of *Bacillus subtilis* crude extract against first instar larvae of *Spodoptera littoralis* and *Spodoptera frugiperda* under laboratory conditions

B.s **crude extract conc. (%)**	***Spodoptera littoralis*** **Mortality % based on exposure time**					***Spodoptera frugiperda*** **Mortality % based on exposure time**				
	1st day	**2nd day**	**3rd day**	**5th day**	**7th day**	**1st day**	**2nd day**	**3rd day**	**5th day**	**7th day**
50	33.3	53.3	70	90	93.3	23.3	40	46.7	50	63.3
25	16.1	46.7	66.7	83.3	86.7	20.7	34.5	37.9	43.3	55.2
12.	6.7	20	60	73.3	76.7	13.3	20	30	33.3	50
6.25	3.1	12.5	34.4	53.1	68.8	13	17.1	21.7	30.4	47.8
LC_{50}	94.25	47.97	10.95	4.98	2.43	140.38	93.05	63.17	51.38	10.23
Control	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

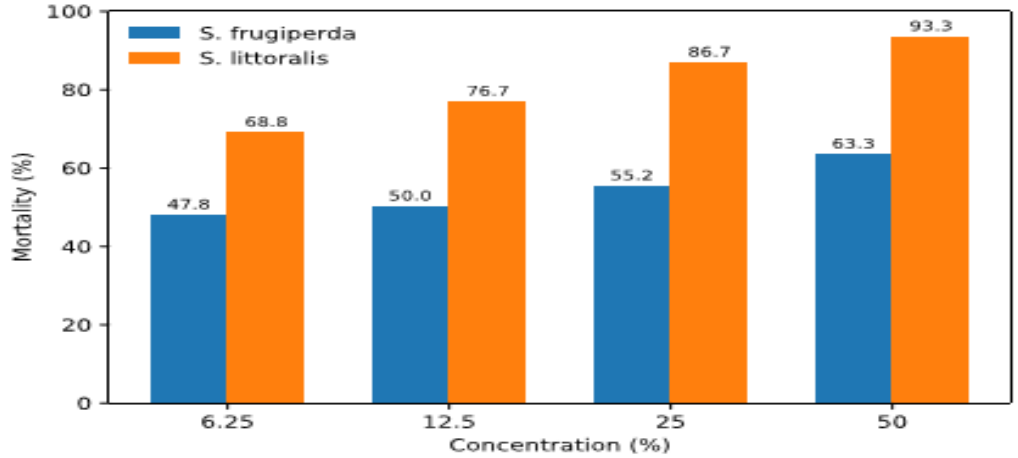

Fig. 3. Cumulative mortality percentage after 7 days of mortality response of the first instar larvae of *S. littoralis* and *S. frugiperda* to *Bacillus subtilis* crude extract after7 days under laboratory conditions

The bacterial isolate was *in vitro* tested for its capacity to antagonize the selected fungal pathogens. *B. subtilis* and pathogens were paired in dual culture at different times (antagonist same

time with the pathogen, 24 h. before the inoculation of the pathogen). Results showed that antagonist and pathogens were paired in dual culture at different times (antagonist at the same time as the pathogen, 24 h. before the inoculation of the pathogen). Results showed that *B. subtilis* was found to have the capacity to suppress the fungal pathogens. *B. subtilis* was more efficient in reducing the growth of phytopathogenic fungi when the bacterial bioagent was inoculated 24 h before fungi inoculation. This agrees with **Gwa and Abdulkadir (2017),** who believed that there are no biological control agents that have enough competitive ability to displace a pathogen that has already established itself on the substrate. The antagonistic microorganisms that cause a reduction in fungal growth by producing a clear zone may have released toxic metabolic substances into the medium **(Akhtar, 1982).**

Bacillus subtilis have demonstrated antagonistic activity against phytopathogenic fungi through several modes of action, its able to secrete different potent antimicrobials such as lipopeptides, specifically the Surfactin, Iturin, and Fengycin families. These metabolites are amphiphilic in nature, allowing them to intercalate into the fungal lipid bilayer. This leads to pore formation, leakage of intracellular ions and eventually programmed cell death of the pathogen. Our findings of a clear "zone of inhibition *in vitro* strongly support the diffusion of these metabolites **(Romero *et al*., 2007).** Also *B. subtilis* can secreate extracellular lytic enzymes, such as beta-1,3-glucanases and chitinases. Since chitin and glucan are the structural backbone of fungal cell walls (**Ajuna *et al*., 2023)**

The present study demonstrated that *B. subtilis*, applied either as a whole culture or as a crude extract, exhibited significant toxicological activity against the first instar larvae of *S. littoralis* and *S. frugiperda*. However, the bacterial culture consistently showed higher larvicidal efficacy than its crude extract alone, indicating that live cells and/or unstable metabolites present in the culture play a crucial role in enhancing insecticidal activity. This observation agrees with previous reports highlighting the superior bio-efficacy of whole microbial cultures compared to isolated extracts due to the continuous production of bioactive metabolites and synergistic interactions among compounds **(Isman, 2006; Senthil-Nathan, 2013)**.

The progressive increase in larval mortality with prolonged exposure time and the corresponding decline in LC_{50} values indicate a cumulative and chronic mode of action rather than an acute toxic effect. Such time-dependent toxicity is characteristic of microbial and metabolite-based insecticides, which often require ingestion and metabolic interaction within the insect midgut to exert lethal effects **(Bloomquist, 2009)**. The higher susceptibility of *S. littoralis* compared with *S. frugiperda* observed in this study may be attributed to interspecific differences in midgut physiology, detoxification enzyme activity, and feeding behavior, as previously reported for lepidopteran pests exposed to microbial toxins **(Li *et al*., 2007).**

The higher efficacy of *B. subtilis* culture compared to its crude extract suggests that synergistic interactions among metabolites, continuous metabolite production, and possible gut colonization collectively enhance insecticidal activity. Similar findings have been reported for other microbial biocontrol agents, where the combination of multiple bioactive compounds results in multi-target toxicity and reduces the likelihood of resistance development **(Koul *et al*., 2004; Senthil-Nathan, 2013)**.

Overall, the results of this study indicate that *Bacillus subtilis* exerts its toxicological effects against *S. littoralis* and *S. frugiperda* through multiple complementary mechanisms, including membrane disruption, enzyme inhibition, neurotoxicity, and behavioral interference. This multi-mode action, combined with time-dependent toxicity, highlights the potential of *B. subtilis* culture and its metabolites as promising eco-friendly alternatives or supplements to conventional chemical insecticides for the management of *Spodoptera* species.

3.4.GC–MS characterization of bioactive metabolites produced by *Bacillus subtilis*

The GC-MS profiling of *Bacillus subtilis* extract revealed a complex mixture of bioactive metabolites, including fatty acids, esters, sulfur-containing compounds, and nitrogenous derivatives. Many of these metabolites have been previously reported to exhibit toxic activity against phytopathogenic fungi (*Rhizoctonia solani* and *Fusarium oxysporum*), as well as larvicidal effects against insect pests such as *Spodoptera littoralis* and *Spodoptera frugiperda***,** as shown in Table (5)**.**

Table 5. GC-MS analysis of bioactive metabolites of crude extract produced by *Bacillus subtilis*

`Peak No.	*RT	Components name	Area%	Molecular formula	Molecular weight	Library
1	6.79	Glucuronamide	6.82	C6H11NO6	193	mainlib
2	14.99	Benzeneacetic acid	20.14	C12H18O2Si	222	replib
3	26.12	Hexahydrofarnesol	0.47	C15H32O	228	replib
4	26.34	Myristyl Alcohol	0.39	C14H30O	214	WileyRegistry8e
5	26.55	Oleic Acid	0.73	C18H34O2	282	replib
6	27.59	1-Dodecanol, 3,7,11-Trimethyl	2.03	C15H32O	228	WileyRegistry8e
7	28.89	Hexadecanoic Acid	10.00	C19H40O2Si	328	WileyRegistry8e
8	31.88	2-Trimethylsiloxy-6-hexadecenoic acid, methyl ester	1.63	C20H40O3	356	mainlib
9	31.96	5,8,11-Eicosatrienoic acid, (Z)-, TMS derivative	1.64	C23H42O2Si	378	mainlib
10	32.42	Linolenic Acid	1.56	C27H52O4Si2	496	WileyRegistry8e
11	36.64	Cedran-diol, (8S,14)-	0.94	C15H26O2	238	mainlib

*****Retention time

3.4.1.Toxic effects of *Bacillus subtilis* crude extract against phytopathogenic fungi and insect pests

GC–MS analysis of the crude culture extract of *Bacillus subtilis* revealed a complex mixture of fatty acids, fatty alcohols, terpenoid-related compounds, and aromatic acid derivatives. Such chemical diversity is characteristic of *Bacillus* secondary metabolites and is closely associated with broad-spectrum antimicrobial and insecticidal activities **(Ongena & Jacques, 2008)**.

3.4.1.1. Antifungal activity against phytopathogenic fungi

Fatty acids and their derivatives, including oleic acid, linolenic acid, hexadecanoic acid derivatives, and eicosatrienoic acid derivatives, constituted a major portion of the detected metabolites. Unsaturated fatty acids are known to inhibit phytopathogenic fungi by disrupting plasma membrane integrity, increasing permeability, and causing leakage of essential cellular components, ultimately suppressing mycelial growth and spore germination **(Desbois & Smith, 2010)**. This mode of action is particularly effective against soil-borne pathogens such as *Fusarium solani*, *Fusarium oxysporum*, *Rhizoctonia solani*, and *Sclerotium rolfsii*.

The dominant presence of benzeneacetic acid, trimethylsil ester suggests a significant contribution of aromatic organic acids to the antifungal activity of the crude extract. Phenylacetic acid and related derivatives produced by *Bacillus* spp. have been reported to strongly inhibit *Fusarium* spp. by interfering with fungal respiration, enzyme activity, and hyphal development **(Dheepa *et al*., 2016; Zhang *et al*., 2018).**

Long-chain fatty alcohols such as hexahydrofarnesol, myristyl alcohol, and 1-dodecanol, 3,7,11-trimethyl also contribute to antifungal efficacy. These compounds are known to integrate into fungal lipid bilayers, alter membrane fluidity, and inhibit ergosterol biosynthesis, leading to reduced fungal growth and pathogenicity **(Avis & Bélanger, 2001).** Additionally, terpenoid-related compounds like cedran-diol may induce oxidative stress and mitochondrial dysfunction in fungal cells, enhancing the overall antifungal effect **(Gao *et al*., 2019)**.

3.4.1.2. Insecticidal activity against *Spodoptera littoralis* and *Spodoptera frugiperda*

The insecticidal activity of the *B. subtilis* crude extract against *S. littoralis* and *S. frugiperda* can be largely attributed to its fatty acids, fatty alcohols, and terpenoid constituents. Free fatty acids such as oleic, linolenic, and γ-linolenic acids have been reported to exhibit contact toxicity and antifeedant effects against lepidopteran larvae by disrupting cuticular lipids and damaging midgut epithelial cells **(Isman, 2020)**.

Terpenoid alcohols, particularly hexahydrofarnesol, are structurally related to juvenile hormone analogs and may interfere with insect endocrine regulation. Such compounds can cause abnormal molting, delayed larval development, and reduced pupation success in *Spodoptera* species **(Tunaz & Uygun, 2004)**. Fatty alcohols like myristyl alcohol further contribute to toxicity by affecting neuromuscular coordination and energy metabolism in insect larvae **(Pavela, 2016).**

Aromatic acid derivatives, including benzene acetic acid, may also play a role as feeding deterrents and metabolic inhibitors, reducing larval growth and survival. The combined action of these compounds likely results in synergistic toxicity, enhancing larval mortality and developmental disruption in both *S. littoralis* and *S. frugiperda* **(Isman, 2020)**. Overall mode of action and biocontrol potential, the biological activity of the *B. subtilis* crude extract is best explained by a multi-target mode of action, arising from the synergistic interaction of membrane-active fatty acids, hormone-disrupting terpenoid, and growth-inhibitory aromatic acids. Such synergism is a well-recognized advantage of microbial biocontrol agents, reducing the likelihood of resistance development and increasing efficacy against a wide range of plant pathogens and insect pests **(Raaijmakers *et al*., 2006; Ongena & Jacques, 2008)**.

Conclusion

The results highlights *Bacillus subtilis* as a potent eco-friendly biocontrol agent exhibiting broad-spectrum activity against both phytopathogenic fungi and lepidopteran insect pests. Its superior efficacy in culture form, along with multi-mechanistic and time-dependent toxicity, supports its promising application in sustainable integrated pest and disease management (IPM) programs as an alternative to conventional chemical pesticides.

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الإمكانات الحيوية لبكتيريا *Bacillus subtilis* في مكافحة الفطريات الممرضة للنباتات وأنواع *Spodoptera*: تقييم سمي وتحليل باستخدام الكروماتوغرافيا الغازية–مطيافية الكتلة(GC–MS)

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الملخص العربى:

تعد الفطريات الممرضة للنباتات والآفات الحشرية التابعة لرتبة حرشفية الأجنحة من أخطر العوامل المقيدة للإنتاج الزراعي عالميا، حيث تسهم في إحداث خسائر اقتصادية جسيمة تهدد الأمن الغذائي. وقد أدى الاعتماد المفرط على المبيدات الكيميائية إلى ظهور سلالات مقاومة، وتفاقم مشكلات التلوث البيئي، وزيادة المخاطر الصحية على الإنسان والكائنات غير المستهدفة، الأمر الذي عزز التوجه نحو استراتيجيات المكافحة الحيوية كبدائل مستدامة وآمنة بيئيا. تهدف الدراسة الحالية إلى تقييم الكفاءة الحيوية للمزرعة البكتيرية لبكتيريا *Bacillus subtilis* ومستخلصها الخام كمعاملات مكافحة حيوية ضد أهم الفطريات الممرضة المنقولة بالتربة، وهي *Fusarium solani* و *Fusarium oxysporum* و*Rhizoctonia solani* و*Sclerotium rolfsii*، بالإضافة إلى الآفتين الحشريتين ذواتي الأهمية الاقتصادية *Spodoptera littoralis* و*Spodoptera frugiperda* تحت الظروف المعملية. تم تقدير النشاط التضادي ضد الفطريات باستخدام تقنية الزراعة المزدوجة وتقنية الغذاء المسمم، في حين جرى تقييم الكفاءة الإبادية ضد يرقات العمر الأول لأنواع *Spodoptera* من خلال اختبارات حيوية معتمدة على الزمن والتركيز. وأظهرت النتائج قدرة تضادية مرتفعة للمزرعة البكتيرية ضد الفطريات المختبرة، لا سيما *R. solani* و*F. oxysporum*، مع زيادة ملحوظة في نسبة التثبيط عند تلقيح البكتيريا قبل إدخال الممرض الفطري، مما يؤكد أهمية الأسبقية الزمنية في تعزيز الفاعلية الحيوية. أحدث المستخلص الخام المستخلص بأسيتات الإيثيل انخفاضا معنويا في نمو الميسيليوم الفطري، حيث بلغت قيم EC_{50} نحو 924.6 و1574.8 جزء في المليون ضد *R. solani* و *F. oxysporum* على التوالي، مما يشير إلى حساسية متفاوتة بين الأنواع الفطرية. وعلى الصعيد الحشري، أظهرت المزرعة البكتيرية فاعلية إبادية تفوقت بوضوح على المستخلص الخام ضد كل من *S. littoralis* و*S. frugiperda*، مع تزايد معدلات النفوق بمرور الزمن وانخفاض قيم LC_{50} مع إطالة فترة التعرض، بما يعكس نمط تأثير تراكمي يعتمد على التفاعل الأيضي داخل اليرقات. كشف تحليل الكروماتوغرافيا الغازية–مطيافية الكتلة (GC–MS) عن تنوع ملحوظ في المركبات الأيضية الحيوية الفعالة، شملت الأحماض الدهنية والكحولات الدهنية والمركبات التربينويدية ومشتقات الأحماض العطرية، وهي مركبات معروفة بقدرتها على إحداث اضطراب في سلامة الأغشية الخلوية، والتداخل في المسارات الأيضية، وإحداث اختلال في التنظيم الهرموني لكل من الفطريات والحشرات. تؤكد النتائج أن *Bacillus subtilis* تمثل عامل مكافحة حيوية واعدا وصديقا للبيئة يتميز بنشاط مزدوج واسع الطيف ضد الفطريات الممرضة للنباتات والآفات حرشفية الأجنحة، مما يدعم إمكانية إدماجها ضمن برامج الإدارة المتكاملة للآفات والأمراض الزراعية كبديل مستدام للمبيدات الكيميائية التقليدية.

الكلمات الدالة: المكافحة الحيوية ، الفطريات الممرضة للنبات ، آفات حشرية ، بكتريا *Bacillus subtilis*