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RESEARCH ARTICLE

The Antagonistic *Bacillus paralicheniformis* SHG3 for the Biocontrol of Some Phytopathogens

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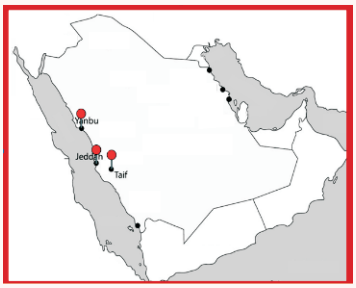
Study Area: Jeddah, Saudi Arabia
Coordinates: 21°32′36″N; 39°10′22″E

Key words: Antifungal, *Fusarium* sp., *Bacillus* sp., Toxicity, Chitinase



Study sites

Abstract

Pathogenic fungi damaged important crops cultivated worldwide. Many plant growth-promoting bacteria can be used to inhibit many pathogens. This study aimed to isolate and identify a selected bacterial isolate to inhibit some pathogenic fungi. Out of 55 bacterial isolates obtained on starch nitrate agar, three isolates showed biocontrol activities against *Fusarium oxysporum* and the chitinolytic index ranged from 1.7-3.1. Isolate SHG3 showed excellent antagonistic activity against *F.oxisporium*, thus it was identified and screened for antagonistic activity against some plant pathogens. The highest antifungal activity was detected against *F.oxysporum*, *Curvularia microspora, C.akaii* and *Rhizoctonia solani* while the highest antibacterial activities were against *Pseudomonas syringae, P.corrugata* and *Pectobacterium carotovorum*. Using morphological and physiological characters in addition to 16Sr RNA, the isolate SHG3 was identified as *Bacillus paralicheniformis* with a similarity level of 97% with *B.paralicheniformis* KY694465.1. The culture filtrate showed no toxicity against *Artimia salina* as a test organism.

Introduction

Plants in their environment face potential pathogens such as fungi, bacteria, viruses and nematodes which led to losses in crop yield. Soil-borne pathogens enter via roots and leaves and cause diseases of seedlings, roots, leaves or stems. The strong disease led to disruption of the water and minerals uptake, transportation, photosynthesis and metabolisms (McGovern, 2015). The use of fungicides to inhibit the attacks of these pathogens was harmful to the environment and human health. Further, fungi acquired resistance towards these chemicals which had toxicity to soil microbiome. The use of microorganisms to control plant pathogens is important. The potential use of plant-associated bacteria as biocontrol agents and stimulating plant growth has been described (Compant *et al*., 2005). The growth-promoting bacteria colonise the root surfaces and closely adhering the soil interface to prevent pathogens from infecting plants. The general mechanisms involved in biocontrol are to reduce the population of pathogens at their entry-level and to provide strong growth-promoting activity (Chen *et al*., 2000; Naik *et al*., 2008).

The genus *Bacillus* improves nutrient uptake from soil and has a role in plant protection. They can antagonize and inhibit many plant root pathogens by promoting seedling growth, decreasing stress factors, increase nitrogen fixation and availability of phosphorus and plant growth hormones which improve plant production and soil fertility. They have also been used to biocontrol the most important fungal pathogens like *F.oxysporum* and *R.solani*, and bacterial pathogens, *P.syringae* and *Pectobacterium carotovorum* (Zhang *et al*., 2020). The plant growth promoting bacteria are important sources of bioactive metabolites which control a wide range of phytopathogens. They can be used as biostimulants, biopesticides, bioherbicides, and biological control agents (Marimuthu *et al*., 2020). Several bacteria were screened for their biocontrol capabilities against fungal and bacterial pathogens and salt tolerance activities (Djebaili *et al*., 2021). This study aimed to evaluate the antagonistic activity of *Bacillus paralicheniformis* against some phytopathogens.

Materials and methods

Tested organisms: *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Enterobacter aerogenes, Klebsiella pneumoniae* and *Acinetobacter baumannii* were provided by Maternity and Children's Hospital in Jeddah, Saudi Arabia. The bacterial phytopathogens, *Agrobacterium tumefaciens, Pseudomonas syringae,*

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P.corrugate, Pectobacterium carotovorum, Ralstonia solanacearum and *Xanthomonas oryzae* and the tested fungi, *Aspergillus niger, Fusarium oxysporium, F.redolense, Talaromyces* sp., *Curvularia microspora, C.akaii*, *Sclerotinia sclerotiorum* and *Rhizoctonia solani* were obtained from the culture collection of the Microbiological Lab., Jeddah, Saudi Arabia.

Sample collection: nine soil samples (100 g each) were collected in sterile plastic bags from the western region of Saudi Arabia including Alhada area in Taif, Alsharifiat area in Yanbu Al-Nakheel and Jamiah area in Jeddah. Also, three shrimp shell samples were collected from Jeddah fish market in sterile plastic bags and preserved at 4″C until used.

Isolation of bacteria from different collected samples: soil samples were used for bacterial isolation on starch nitrate agar (Shirling & Gottlieb, 1966) using the serial dilution method (Mohseni *et al*., 2013) while shrimp shells were cut into very small parts which were put directly on the agar surface using sterile forceps (Aly *et al*., 2011). All the prepared plates were incubated at 30″C for 48 hrs. Then, the appeared bacterial colonies were selected, purified and preserved on slants of the same medium to be used in the next experiments.

Growth on Chitin agar: shrimp shell was collected, demineralized in 5% HCl, deproteinization with 3% NaOH and the obtained chitin was used to prepare soluble chitin as described by Trachuk *et al*. (1996). All the isolated bacteria were screened for chitinase activity in the chitin agar medium (Kim *et al*., 2003). The chitinolytic index also varies, from weak to strong and are considered strong if the index is more than 2 (Chasanah *et al*., 2009; Hardoko *et al*., 2020).

$$\text{Chitinolytic index} = \frac{\text{diameter of zone formed}}{\text{diameter of the colony.}}$$

Antifungal activities of bacterial isolates using dual-plate confrontation assay: the target fungi were cultured on potato dextrose agar g/l- (fresh potato 200.0, starch 20.0, agar 13.0, pH 7). The antagonism of all bacterial isolates was checked with respect to the ability to suppress *Fusarium oxysporum* growth. Antifungal bioassay was performed with the dual culture on PDA medium where the selected bacterium was inoculated as a single streak across the middle of the plate, the plates were inoculated for 5 days and the tested fungus was inoculated on both sides, clear zone indicated that the bacteria produced antifungal substance inhibited fungi. The most active isolate SHG3 was selected to be examined against different plant pathogens using modified dual-plate confrontation assays and antagonistic activity of the bacterium SHG3 was recorded (Kong *et al*., 2020).

The inhibition rate (%) = Colony diameter of the control – Colony diameter of the tested × 100% /Control diameter

The activity of the tested bacterium against some bacterial pathogens: the antimicrobial activities of the isolate SHG3 were detected using agar well diffusion assay (Sananthaya & Thirabunyanon, 2009) against some human pathogenic bacteria (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter aerogenes* and *Acinetobacter baumannii*) and some plant pathogenic bacteria (*Agrobacterium tumefaciens, Pseudomonas syringae, P.corrugate, Pectobacterium carotovorum, Ralstonia solanacearum and Xanthomonas oryzae*). The presence of the inhibition zone around the well indicated positive results.

Extracellular hydrolytic enzyme assays and toxicity: additionally, extracellular hydrolytic enzymes including chitinase, protease, cellulose, and glucanase, which participate in the mechanisms underlying fungal growth inhibition, were measured qualitatively and quantitatively as previously described (Abiala *et al*., 2015; Wang *et al*., 2019; Ali *et al*., 2020). Cytotoxicity of bacterial filtrate extracted with diethyl acetate was determined using brine shrimp lethality test (Aly & Gumgumji, 2011).

Bacterial identification: morphological, physiological and biochemical tests were performed for the identification of the isolate SHG3 at 30°C. These tests include- Gram stain, spore shape, colony morphology, the ability of the strain to hydrolysis of starch, gelatin liquefaction, indole production, hydrolysis of urea, oxidase and catalase (Mishra & Behera, 2008; Tork *et al*., 2010). Resistance to antibiotics was determined using Muller -Hinton agar plates incubated at 30°C for 24hrs (CDC, 2019).

DNA was extracted and 16SrDNA genes of the active isolates were purified and sequenced following the manufacturer's suggested protocols. The sequence was analyzed and compared to 16SrDNA genes in the GenBank databases (Weisberg *et al*., 1991; Xu *et al*., 2021).

Results

Out of nine soil samples and three shrimp shell samples, 55 pure bacterial isolates were obtained on starch nitrate agar. The growth on both Starch Nitrate and Chitin agar was determined and the antagonistic activity against *Fusarium oxisporium* was recorded (Table 1). Three bacterial isolates showed good antagonistic activity against *F.oxisporium*. The isolate SHG3 from the Shrimp shell showed excellent antagonistic activity against *F.oxisporium*. It recorded the highest chitinolytic index (3.1), thus, it was selected for more detailed studies. The isolate SHG3 was examined and morphologically characterized as a long rod, Gram-positive spore-forming bacterium. Colonies are raised, creamy white, mucoid, translucent and about 4 mm in diameter. On chitin agar the cell diameter was 0.5–0.7 mm in diameter and 1.7–3.8 mm long but when cultured on starch nitrate agar larger diameter was obtained. Fig.-1 showed the antagonistic activity of the isolate SHG3 against *F.oxisporium*, Gram stain and resistance to different antibiotics. Morphological, physiological ad biochemical characteristics of the selected isolate SHG3 were recorded in Table 2. The characteristics of the isolate SHG3 were close

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to *Bacillus licheniformis*. Using the molecular technique, it belongs to *B.licheniformis* clustered which had two distinct groups, group 1 with *B.licheniformis* NR_113588 and group 2 consisting of SHG3 and ten other strains (Fig.-2). It could be identified as *B.paralicheniformis* SHG3 with 97% similarities to *B.licheniformis* or *B.sonorensis* on the basis of the phylogenomic analysis.

The selected isolate recorded antagonistic activity (diameter of the inhibition zone) against all the tested bacteria with inhibition zone ranging from 13-37 mm (Table 3). The highest activity was recorded against *Pseudomonas syringae, P.corrugate, Escherichia coli* and *Enterobacter aerogenes*. The results of the antagonistic activity of the isolate SHG3 against some plant fungal pathogens were represented in Table 4. The selected isolate SHG3 recorded excellent antagonistic activity against all the tested fungi with inhibition percentages ranging from 23.9-79.9 % (Fig.-1). The highest activity was recorded against *F.oxysporium* (73.9%), followed by *Rhizoctonia solani* (70.9%), *Fusarium redolense* (60.9 %), *Curvularia akaii* (55.9%), *Aspergillus niger* (53.0%) and *Curvularia khuzestanica* (43.0%). The lowest activity was recorded against *Talaromyces* sp. (29.0%) and *Sclerotinia sclerotiorum* (23.9%).

Table 1. The source of the isolated bacteria, their growth on Starch Nitrate Agar (SNA) and Chitin Agar (CA), the Antagonistic activity (Aa) against *F.oxisporium* and Chitinolytic index (Ci) for each isolate

Isolate	Source	SNA	CA	Aa*	Ci
SHJ3	Jeddah	+++	+	M	1.9
SHR31	Yanbu Al-Nakheel	+++	+	M	1.7
SHG3	Shrimp shell	+++	+	VH	3.1

*Antagonistic activity against Fusarium oxisporium, +++: high growth, ++: moderate growth, M: Moderate, VH: Very high

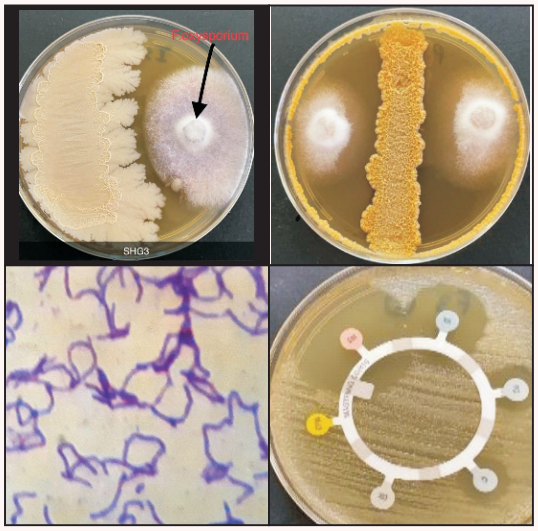


Figure-1: Antagonistic activity of the isolate SHG3 against *Fusarium oxisporium* (A), *F.redolense* (B), Gram stain (C) and resistance to antibiotics (D).

Table 2. Characters of the selected isolate SHG3

Test	Result	Test	Result
Source	Shrimp shell	Starch hydrolysis:	Positive
Colonies color	Beige	Nitrate reduction	Positive
Shaped	Long Bacillus	Use of citrate	Positive
Growth in the presence of NaCl	3-15%	Resistance to Erythromycin	Positive
Gelatin hydrolysis	Positive	Acid production	Positive

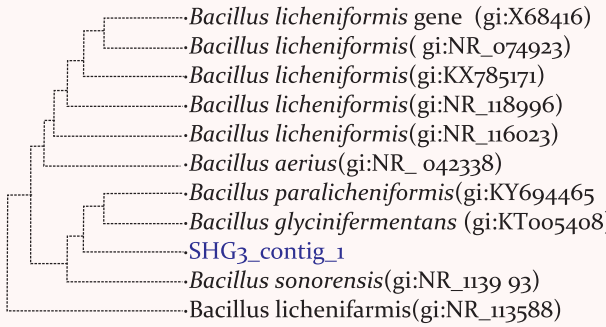


Figure-2: Phylogenetic tree reconstructed from the core genomes of the isolate SHG3

Table-3: The antagonistic activity (diameter of the inhibition zone) of the isolate SHG3

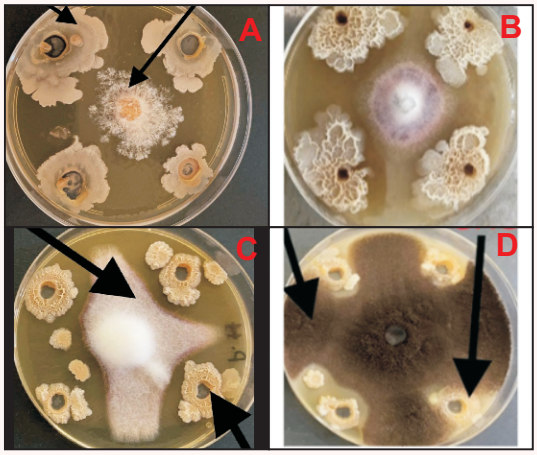
Tested Bacteria	Results	Tested Bacteria	Results
Staphylococcus aureus	18±0.2*	*Agrobacterium tumefaciens*	21±0.8*
Enterobacter aerogenes	29±0.09*	*Pseudomonas syringae*	37±0.8*
Acinetobacter baumannii	29±0.11*	*Pseudomonas corrugata*	33±1.8*
Escherichia coli	29±0.02*	*Pectobacterium carotovorum*	30±0.9*
Klebsiella pneumoniae	24±0.13*	*Ralstonia solanacearum*	23±0.8*
Pseudomonas aeruginosa	20±0.8*	*Xanthomonas oryzae*	13±0.8*

* significant results compared to control (ampicillin, 5 µg/ disc).

Table 4: The antagonistic activity of the isolate SHG3 against some fungi (I%- inhibition percent)

Tested fungi	I%	Tested fungi	I%
Fusarium oxysporium (Control)	73.9±4.03	*Aspergillus niger*	53.0±4.33*
Curvularia akaii	55.9±1.49*	*Rhizoctonia solani*	70.0±1.44*
Curvularia khuzestanica	43.0±9.11*	*Talaromyces* sp.	29.0±2.07*
Fusarium redolense	60.9±2.61*	*Sclerotinia sclerotiorum*	23.9±0.95*

*significant results compared to control.



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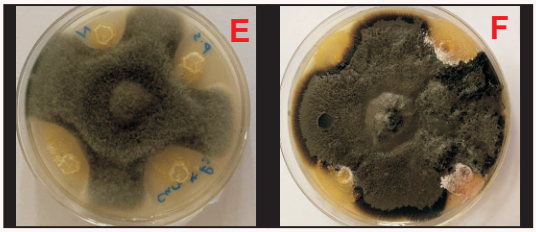

Figure-3: Growth of *Rhizoctonia solani* (A), *Fusarium oxisporium* (B), *F.redolense* (C), *Aspergillus niger* (D), *Curvularia akaii* (E) and *Curvularia khuzestanica* (F) after 7 days in the presence of *Bacillus paralicheniformis*

The selected isolate was active in producing chitinase, protease, cellulose, and glucanase (Fig.-4). High production was recorded for chitinase and protease on solid medium while the moderate show was recorded for glucanase and cellulose. The production was confirmed in a liquid medium where the enzyme activity was recorded as U/ml. It was found that no toxicity was recorded to all tested concentrations up to 800 µg/ml using *Artimia salina* as a test organism, LD50 was more than 800 µg/ml (Table 5).

Table 5. Detection and potential production of some enzyme by isolateSHG3

Enzyme	Liquid medium		Toxicity	
	Absorbance	U/ml	Conc. (µg/ml)	Ld50
Chitinase	1.33	4.33±1.01	100	″ 100
Protease	0.79	5.14±0.91	200	″ 200
Glucanase	0.89	2.99±0.14	400	″ 300
Cellulose	0.88	0.89±0.09	800	″ 400

Discussion:

During growth, plants are subject to different biotic and abiotic stresses like fungal diseases that can be extremely deleterious to the plant by attacking different plant organs. The fungal genera *Fusarium, Sclerotinia, Rhizoctonia*, and *Curvularia* can consider the most harmful diseases for the cultivation process of tomatoes, barley and vegetables (Figueroa *et al*., 2018). Fusarium wilt is an important disease that affects all plant organs and causes 30–70% yield losses and necrosis. After 72 hrs at high humidity and low temperature, the spores germinate and initiate plant infection. The critical stage of infection was when anthesis developed due to the high-level choline and betaine which stimulate fungal growth (Karlsson *et al*., 2021). Also, *Curvularia* belongs genus hyphomycete which causes a wide range of serious plant diseases on economically important crops like barley, maize, rice, sorghum, and wheat (Bengyella *et al*., 2019).

Biocontrol of fungal pathogens by useful bacteria is urgent and a population of chitin-degrading bacteria in soil can attack chitin found in the cell walls of some plant pathogens. Chitin-degrading bacteria produce chitinases like *Bacillus, Paenibacillus* and *Streptomyces* has a potent role to inhibit fungi by degradation of their cell walls (Mizumoto *et al*., 2007; Shimoi *et al*., 2020, Ootsuka *et al*., 2021). Thus, the isolate SHG3 which was isolated from

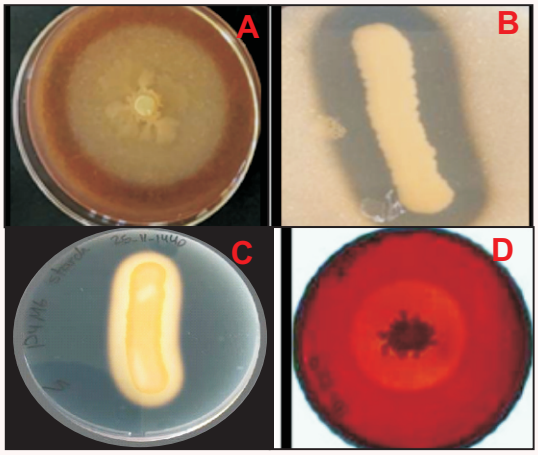

Figure-4: Chitinase (A), protease (B) glucanase (C) and cellulase (D) production by the selected isolate SHG3.

shrimp shells, grow well on chitin agar and was highly antagonistic to the Fusarium was selected for this study. The isolate SHG3 is a Gram-positive rod endospore-forming bacterium. Furthermore, phylogenetic analysis of the 16S rRNA gene indicated that the strain was most closely related to *Bacillus paralicheniformis* KJ-16T (similarity 97.0 %). Similar to this isolate, a novel isolate from fermented soybean food product was isolated and characterized as a novel species of *B.paralicheniformis* (Dunlap *et al*., 2015).

Isolate SHG3 can be utilized as a biocontrol agent to inhibit phytopathogenic fungi. Similar to this result, *Streptomyces albidoflavus* and *Nocardiopsis aegyptica* showed the best biocontrol activities. The diffusible and volatile compounds of these strains showed antifungal and antibacterial activity. Rhizobacteria produce plant growth-promoting materials that increase plant growth and act as biofertilizers or as competitors of plant-pathogenic fungi (Beneduzi *et al*., 2012). The genus *Bacillus* is one of the most active genera that increase plant growth and suppressed many plant phytopathogens due to the production of many antibiotics (Wu *et al*., 2015; Zhao *et al*., 2014).

In agriculture, members of the genus *Bacillus* are reported as soil inoculants for some plants to promote plant growth and biocontrol pathogens that mainly infect the root region. The previous genus produces many secondary compounds with potential applications in medicine and agriculture (Sieber *et al*., 2005). These antibiotics suppressed different plant pathogens due to the production of Bacitracin which acts against filamentous fungi and Paralichenicidin which suppresses plant pathogenic bacteria and fungi (Dunlap *et al*., 2015). From morphological, physiological and molecular characters, the isolate SHG3 was identified as *B.paralicheniformis* SHG3 and is most closely related to *B.licheniformis* and *B.sonorensis*, based on phylogenetic analysis. Similarly, *B.paralicheniformis* MDJK30 was recently isolated and identified from the rhizosphere of the peony in Shandong, China (Wang *et al*., 2019). It showed high antagonistic

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activity to *F.solani* and *B.subtilis*, which indicates the potential application for controlling pathogenic fungi and bacteria. The isolate MDJK30 has the potential to synthesize bioactive secondary metabolites (Medema *et al*., 2011) like siderophores for iron competition and fitness in iron-limited environments. Lantipeptide represents a potential antimicrobial strategy against a range of pathogenic bacteria and fungi (Stein *et al*., 2005). The evolutionary relationship between *B.licheniformis* and *B.paralicheniformis* suggested that the two isolates have occurred during adaptation to different niches and plant-pathogenic bacteria and fungi could be an important driving factor (Hartmann *et al*., 2009). The cells of *B.paralicheniformis* had many genes that had a role in cell metabolisms and related to rhizosphere adaptation (Sun *et al*., 2015). Also, *Bacillus* cells secreted the cyclic lipopeptides to protect host plants from many pathogens. Fengycin prevents a large number of plant pathogens, especially filamentous fungi (Fan *et al*., 2017). Bacitracin and Paralichenicidin are broad-spectrum antibiotics synthesized by several strains of Bacillus (Rey et al., 2004). *B.paralicheniformis* SHG3 possesses several beneficial properties and has the potential to inhibit pathogens and enhance plant growth through different metabolic pathways and utilization of a variety of carbon sources. Thus, It can be developed and commercially used for field application, either alone or as part of microbial consortia to control different phytopathogens. Isolate SHG3 showed excellent antifungal activity which may be due to the production of different lytic enzymes for the degradation of fungal cell walls. Similarly, the actinomycete strain, *Streptomyces exfoliatus* produce three fungal cell-wall lytic enzymes while *Streptomyces violascens* had good biocontrol potential against toxigenic fungi due to the high production of extracellular antifungal enzymes (Leong *et al*., 2004; Choudhary *et al*., 2014, 2015). Also, the active metabolites of the isolate SHG3, extracted using diethyl acetate showed no toxicity using Artimia salina as the test animal. Also, Alshamrani & Aly (2022) used the same technique to detect the toxicity of some plant extracts. In conclusion, the selected isolate which was identified as *B.paralicheniformis* SHM3 possesses several beneficial properties and is a promising gram-positive bacterial isolate, using various energy sources. Thus, it can be developed and commercially used either alone or as part of microbial consortia, for field application to control plant pathogens and promote crop growth. It can be used also as a biocontrol agent for many plant and human pathogens.

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