Biochemical and Molecular Identification of a Native *Bacillus thuringiensis* gv. *cytolyticus* Isolate with Insecticidal Effect Against the Pod Borer Larvae (*Helicoverpa armigera*)

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ABSTRACT

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The aim of this study is to isolate and identify autochthonous bacterial strains with insecticidal effect against the larvae of the tomato moth *Helicoverpa armigera*. Dead and diseased larvae were collected from chili plots in the region of Gotraniya from the governorate of Kairouan, Tunisia. These larvae were used to isolate eight bacterial strains named Hr1 to Hr8. The bacterial isolates were characterized by macroscopic and microscopic observations and 16S rRNA sequencing. Hr1, Hr2, Hr4, Hr5, Hr6, and Hr8 were identified as *Bacillus* spp., Hr3 as *Staphylococcus* sp., and Hr7 as *Enterobacter* sp. Their insecticidal activity was evaluated against third-instar larvae of *H. armigera*. A Hr1 bacterial isolate showed an insecticidal effect against *H. armigera* larvae, causing 60% larval mortality four days post-treatment at a dose of 1.8×10^9 CFU/ml. Based on further characterization studies, Hr1 was identified as *Bacillus thuringiensis* gv. *cytolyticus* following Biolog biochemical tests and multi-locus sequence analyses studies based on sequencing of seven housekeeping genes.

Keywords: *Bacillus thuringiensis* gv. *cytolyticus*, biological control, Biolog, entomopathogenic bacteria, *Helicoverpa armigera*, multi-locus sequence analysis, 16S rRNA sequencing

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The pod borer, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae), is a cosmopolitan Lepidopteran pest reported in several crop species, including cotton, tomato, chickpea, maize, numerous legumes, ornamental plants, and fruit trees (Razmjou et al. 2014; Sun et al. 2004). This insect has a large geographic distribution across all continents Leite et

al. 2014; Jones et al. 2019. It has recently invaded South and Central America and threatens to expand to North America (Kriticos et al. 2015; Tembrock et al. 2020). Infested plants were reported in maize and soybean fields in Serbia and Montenegro and in sunflower fields in Hungary, Serbia, and Montenegro between 2003 and 2004 (Horváth et al. 2004; Sekulić et al. 2004). Over the same period, exceptionally severe damage was reported in many fields and greenhouse crops in Italy (Sannino et al. 2004). In Tunisia, the damage of this pest was only reported in open field tomatoes and in greenhouse crops for several years (Boukhris-Bouhachem et al. 2007). However, in 2017, damages of *H. armigera* attacks have also been observed in chickpea plants in Tunisia (Bouslama et al. 2019). Severe damage is caused by *H. armigera* larvae, particularly to reproductive organs (Nibouche et al. 2007; Timsina et al. 2007). High movability, fertility, and facultative diapause are features enabling the pod borer to adapt to environmental changes and survive in various habitats (Riley et al. 1992; Zhou et al. 2000; Naseri et al. 2009; Razmjou et al. 2014).

Economic losses can be observed by yields cutback and the increase in the cost of monitoring and insecticides used to control *H. armigera* populations. These losses have been globally evaluated to be five billion USD (USDA 2015). Moreover, this insect has evolved resistance to a large variety of insecticides: pyrethroids, organochlorines, organophosphates, spinosad, and carbamates (Qayyumet al. 2015a; Joußßen and Heckel 2016; Bird 2017). Unfortunately, Tunisian farmers depend mainly on chemical control (Tunisian Ministry of Agriculture, Water Resources and Fisheries 2020) as the use of genetically engineered crops that produce *Bacillus thuringiensis* toxins (i. GMO Bt crops) is forbidden. Furthermore, taking the health and environmental effects of chemical pesticides into account, it was necessary to seek alternative strategies for the pod borer control. Biocontrol studies using entomopathogenic organisms like fungi, viruses, nematodes, bacteria, and natural plant products have shown some potential to the control of *H. armigera* (Cherry et al. 2000; Pawaret al. 1986; Sehgal and Ujagir 1990; Romeis and Shanower 1996). Several microorganisms have been screened against *H. armigera* for their efficacy and their environmental impact, including human safety, such as *Metarhizium anisopliae* (Wakil et al. 2013), *Bacillus thuringiensis* (Wakil et al. 2013; Qayyum et al. 2015b; Dammak et al. 2016), nuclear polyhedrosis virus (Qayyum et al. 2015b; Ben Tiba et al. 2022), and granulosis virus (Laarif et al. 2003, Ben Tiba et al. 2018). To date, in Tunisia, registered bio-insecticides are limited, except for one insecticide based on nuclear polyhedrosis virus (Ministry of Agriculture, Water Resources and Fisheries 2020), so the development of effective biocontrol agents are necessary for effective control of plant pests.

In view of this, the present work was carried out to study the cause of epizootic diseases caused by native microorganisms and that can lead to illness or death in natural insect populations. Once isolated, identified, and tested against insect pests, these microorganisms can be used as microbiological control agents in integrated pest management programs. Dead and diseased pod borer larvae were used to isolate several bacteria that were screened for their insecticidal potential against healthy *H. armigera* larvae. Among them, the Hr1 isolate showed a significant insecticidal potential against different instar larvae and was then identified as *Bacillus thuringiensis* gv.

cytolyticus by a polyphasic approach including sequencing of seven housekeeping genes.

MATERIALS AND METHODS

Larva collection and bacterial isolation.

Several collections of larvae were made on chili field crops in the region of Gotraniya (governorate of Kairouan, Tunisia) in order to set up rearing of the pod borer that will be useful in bioassays. The collected larvae were fed with artificial diet (Armes et al. 1992) under controlled laboratory conditions with 16 h of light at a temperature of 25±2 °C and relative humidity of 70±5 %.

Thirty deceased and ailing caterpillars demonstrating characteristic disease manifestations such as no feeding, slow motion, and color changes on the body were collected from an untreated chili field on a farm in the region of Gotraniya (35.730587 N, 10.059041 O). Bacteria were isolated from larvae as described by Çakici et al. (2014), and ten batches, each composed of three dead and three diseased larvae, were homogenized in 10 ml of liquid Lysogenic Broth (LB) medium (10 g of peptone, 5 g of NaCl and 5 g of yeast extract per liter of distilled water, pH adjusted to 7.1). 100 µl of homogenized larvae were plated on solid LB medium. After overnight incubation at 28 °C of the Petri dishes, the isolates were separated based on their colony color and morphology. Purified inoculums of the isolated bacterial strains were stored at -20 °C.

16S rRNA sequencing of bacterial isolates.

Partial sequencing of approximately 1,350 bp of the 16S rRNA gene was used for initial identification of the bacterial isolates. Total genomic DNA extraction was performed according to the standard protocol of Wilson (1997).

PCR amplification of the 16S rRNA genes was performed using pairs of universal primers (Winker and Woese 1991). Reactions were routinely performed in total volumes of 50 µl volume containing 10 µl of 5X *MyTaq* Reaction Buffer, 1 µl (50 ng) of the template, 1 µl (20 µM) of each primer, 1 µl of *MyTaq* DNA Polymerase (Bioline, France), and 36 µl of dH_2O. PCR conditions were as follows: 1 min at 95 °C for initial denaturation of the template DNA, 35 amplification cycles of 15 s at 95 °C, 15 s at 56 °C, 10 s at 72 °C, and 1 min at 72 °C for the final extension. Amplification was carried out in a thermocycler (Biometra, T professional thermocycler, Goettingen, Germany). A quantity of 1.0% agarose gels colored with ethidium bromide were used to separate and view (under UV light) the PCR products. An ABI BigDye Terminator v3.1 Cycle Sequencing kit (Applied Foster City, CA) was used for DNA sequencing in a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, Hercules, CA). Searches were performed with the obtained sequences in the NCBI GenBank database (Altschul et al. 1990) and the Ribosomal Database Project (RDP) at Michigan State University (Michigan State University 2021), respectively. Sequences 1.300 to 1.400 bp in length were used for BLAST analysis with a confidence threshold ≥ 0.05.

Bacterial culture conditions.

Eight bacterial isolates named Hr1 to Hr8 were used in the screening test. The single colonies of bacterial isolates were inoculated into 10 ml of nutrient broth and incubated at 30 °C overnight or for two days in a rotating shaker at 200 rpm. After incubation, the bacterial concentration of each suspension was determined by decimal dilutions and plating on LB agar medium to determine

colony-forming units (CFUs). The concentration of each suspension was adjusted to 1.8×10^9 CFU/ml by adding appropriate amounts of liquid LB medium to the different suspensions to obtain the inoculums used in the screening test. The final bacterial inoculums were prepared as described by Bouslama et al. (2020).

Bioassays.

Spinosad-based insecticide (Tracer 240 SC®) at a concentration of 300 ppm and a PBS 1X solution were used as positive and negative controls, respectively. Tracer is the only insecticide authorized in organic farming against moths in Tunisia, hence our choice (Centre Technique de l'Agriculture Biologique 2021).

Third instar larvae picked from the same colony of a rearing maintained at the laboratory conditions were used for the bioassays. Artificial diet cubes (1 g) were prepared, inoculated with 1 ml of each bacterial suspension, and used for the assay. Caterpillars were arranged in Petri dishes individually to avert cannibalism.

Hr1 was the most effective bacterium against *H. armigera* larvae during the screening test. In order to determine the optimal concentration of Hr1 against different larval instars of *H. armigera*, five different bacterial concentrations were tested (from 1.8×10^9 CFU/mL to 1.8×10^5 CFU/ml). Negative and positive controls were the same as the screening test. Bioassays were performed under controlled laboratory conditions under 16 h of light, a temperature of 25±2 °C, and 70±5 % relative humidity.

For the screening test, the different treatments were tested on 300 healthy third instar larvae of *H. armigera* following a full random device: ten larvae per treatment and three replicates of each treatment group. Dead caterpillars were counted daily for ten days and the percentage of total survival rate produced by each treatment was calculated. The experiment was repeated four times. The dose response test was performed on two batches of 210 first-instar and third-instar larvae following a full random device: ten larvae per treatment and three replicates of each treatment group. Dead caterpillars were counted daily for four days, and the percentage of total mortality produced by each dose was calculated and corrected using the Abbott formula (1925). The experiment was also repeated four times.

Statistical analysis.

Statistical analysis of the different bioassays was performed using the software SPSS 25. First, Shapiro-Wilk and Kolmogrov-Smirnov tests were performed on both data of the percentage of total survival rate and data of the percentage of total mortality produced by the screening test and the dose response test, respectively. Data not satisfying the assumption of the mentioned parametric tests were transformed (Templeton 2011). Analysis of variance (ANOVA) and a Student, Newman, and Keuls (SNK) multiple range tests were then used to analyze the data of total percentage of survival rate and total percentage of mortality produced by the screening test and the dose response test, respectively. In order to detect the most sensitive larval stage to different doses of Hr1, data of percentage of total mortality of first and third instar larvae was further tested using a Student test (t-test) for independent samples.

Biolog test.

The bacterial isolate Hr1 was further characterized by a polyphasic approach including a Biolog biochemical test and multi-locus sequence analyses studies based on sequencing of seven housekeeping genes. Biolog GEN III microplates 1030 (Biolog Inc., Hayward,

CA, USA) was used for the biochemical characterization. The isolate was identified by analyzing the similarity of the metabolic fingerprints between Hr1 and standard strains in the kinetic database using Biolog Retrospect 2.0 Data management software.

Multi Locus Sequence Analysis (MLSA).

An optimized Multi Locus Sequence Analysis (MLSA) scheme developed by Tourasse et al. (2006) was used for further identification of the bacterial isolate Hr1. The methodology consists in the sequencing of 300 to 700 bp housekeeping genes haphazardly scattered within the bacterial chromosome (Sorokin et al. 2006). The total fragment length used for the MLSA scheme was 2,658 bp. The selected genes were *adk* (adenylate kinase), *glpT* (glycerol-3-phosphate permease), *panC* (pantothenate synthetase), *ccpA* (catabolite control protein A), *glpF* (glycerol uptake facilitator protein), *pycA* (pyruvate carboxylase), and *pta* (phosphate acetyltransferase). The primers of the different genes are listed in Table A1 of the Appendix.

A Nucleospin microbial DNA kit (Macherey-Nagel, Germany) was used for total genomic DNA extraction. PCR reactions were done in total volumes of 50 µl formed by 5 µl of 10X PCR Reaction Buffer, 4 µl (2 mM) of dNTP mix, 3 µl (25 nM) of $MgCl_2$ 1 µl (50 ng) of the template, 5 µl (20 µM) of each primer, 0.25 µl of *Taq* DNA Polymerase, and 29.75 µl of dH_2O. Amplification was carried out in a thermocycler (Bioemetra, T gradient thermocycler, Biolabo Scientific Instruments SA, Switzerland). PCR conditions were conducted in this manner: 4 min at 94 °C for initial denaturation of the DNA template, 30 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, and 7 min at 72 °C for the final extension. A quantity of 1.0% agarose gels colored with ethidium bromide were used to separate and view (under UV light) the PCR products. Sequencing of the PCR products was performed by Genoscreen (Lille, France). Hr1 sequences were analyzed against others belonging to the *Bacillus cereus* group and determined by the Tourasse-Helgasson scheme (Tourasse et al. 2006). These sequences were downloaded from an online database of the University of Oslo. In total, 315 sequences of each gene were downloaded from the database. These genes were aligned with each gene of the bacterial isolate Hr1 using Mega X (Kumar et al. 2018). Aligned sequences of each gene were concatenated in a single file using Seaview version 4. The neighbor-joining method (Saitou and Nei 1987) was used to construct a super-tree from the generated file. The evolutionary history of taxa was represented with a bootstrap consensus tree deduced from 1000 replicates; branches with bootstrap values under 70% were eliminated (Felsenstein 1985). The Tamura's 3-parameter method (Tamura 1992) was used to evaluate the evolutionary distances that were represented with units expressing the number of base substitutions per site. A gamma distribution model with a shape parameter equal to one was used to model the rate of variation among sites. A number of 316 nucleotide sequences was analyzed. The pairwise deletion option was used to remove all ambiguous positions from the sequences. The final data set contains a total of 2,668 positions. The MEGA X software (Kumar et al. 2018) was used to conduct evolutionary analysis. The iTOL v3 webserver3 (Letunic and Bork 2016) was used to display clusters and species with the phylogenetic tree.

RESULTS

Bacterial isolation and characterization.

Dead and diseased *H. armigera* larvae collected from an untreated chili pepper field were screened for entomopathogenic bacteria. Eight bacteria were isolated and named from Hr1 to Hr8. The Gram staining results and the morphological properties of the isolates after growth on nutrient agar are given in Table 1.

Table 1. Morphological characteristics of the bacterial isolates

Isolate	Test								
	CC	**SC**	**S (mm)**	**M**	**E**	**S**	**SB**	**GS**	**SP**
Hr1	Brick red	Undulate	2	Wavy	Flat	Rough	Rod	+	+
Hr2	White	Undulate	1	Irregular	Raised	Mucoid	Rod	+	+
Hr3	Golden brown	Pinhead	1	Entire	Convex	Smooth	Coccus	+	-
Hr4	White	Undulate	1	Irregular	Raised	Mucoid	Rod	+	+
Hr5	Cream-white	Round	2	Entire	Raised	Mucoid	Rod	+	+
Hr6	White	Undulate	2	Irregular	Raised	Mucoid	Rod	+	+
Hr7	Cream	Round	1	Entire	Raised	Mucoid	Rod	-	-
Hr8	Yellow-white	Round	2	Entire	Raised	Mucoid	Rod	+	+

*CC: Colony color, SC: Shape of colonies, M: Margin, E: Elevation, S: Surface, SB: Shape of bacteria, GS: Gram staining, SP: Spore presence.

For the determination of the taxonomic affiliation of the different isolates, 1,367 to 1,451 bp of the 16S rRNA gene were sequenced. Hr1 was identified as *Bacillus* sp. belonging to the *B. cereus* group; Hr2, Hr4, Hr5, Hr6, and Hr8 were identified as *Bacillus* spp. belonging to the *B. pumilus* group; Hr3 as *Staphylococcus* sp.; and Hr7 as *Enterobacter* sp. (Table 2).

Table 2. Identification of the 8 bacteria isolated from *H. armigera* larvae based on 16S rRNA sequence similarity with NCBI strains and RDP taxonomic assignment

Isolate	Sequence length (bp)	Isolate GenBank accessions	Sequence similarity with NCBI			Taxonomic assignment (RDP)
			Species name	GenBank accessions	Similarity (%)	
Hr1	1451	MN822194	*Bacillus thuringiensis* *Bacillus cereus* *Bacillus toyonensis* *Bacillus paranthracis*	NR_043403.1 NR_074540.1 NR_121761.1 NR_157728.1	100 100 100 100	Genus *Bacillus*
Hr2	1426	MN822226	*Bacillus safensis*	NR_113945.1	98	Genus *Bacillus*
Hr3	1437	MN822244	*Staphylococcus saprophiticus*	NR_115607.1	100	Genus *Staphylococcus*
Hr4	1441	MN822245	*Bacillus safensis*	NR_113945.1	100	Genus *Bacillus*
Hr5	1442	MN822246	*Bacillus australimaris*	NR_148787.1	98	Genus *Bacillus*
Hr6	1449	MN822717	*Bacillus australimaris*	NR_148787.1	99	Genus *Bacillus*
Hr7	1367	MN822296	*Atlanticbacter hermanii*	NR_104940.1	99	Genus *Salmonella*
Hr8	1397	MN822300	*Bacillus safensis*	NR_113945.1	96	Genus *Bacillus*

Screening of bacterial isolates against *H. armigera*.

The eight isolated bacterial strains caused differential mortality to third-instar larvae, compared to one another, to a positive control (Spinosad, a phytosanitary agent), and a negative control (PBS) during the experiment's period (Table A2 of the Appendix). Hr1 was the most effective bacterium against *H. armigera* larvae, causing 65 % of larval mortality after four days of treatment and 99 % of larval mortality after ten days of treatment. Larval mortality of the other bacterial isolates ranged from 0 % to 2 % after four days of treatment and did not exceed 33 % after ten days of treatment (Fig. 1).

A color shift was observed in the larvae infected with Hr1: they became brick red, like Hr1 colonies on nutrient agar (Fig. 2).

A dose-response test was performed with five different concentrations of isolate Hr1 on two batches of *H. armigera* larvae, i.e., one batch of first instars (weight 0.68 ± 0.068 mg) and one batch of third instars (weight 8.4 ± 0.59 mg). Each concentration caused differential mortality on first instars and third instars during the experiment s period (Table A3 of the Appendix). C1 (1.8×10^9 CFU/ml) caused 95.27% mortality within four days of initial infection of first instar larvae and 65% mortality within four days of initial infection of third instar larvae. C2 (1.8×10^8 CFU/ml) caused 54.98% mortality within four days of initial infection of larvae infected at the first larval stage, and 39.99% mortality within four days of initial infection of larvae infected at the third larval stage (Fig. 3). The t-test showed that first instar larvae are more sensitive to treatments with different doses of Hr1 (Fig. 3, Table A4 of the Appendix).

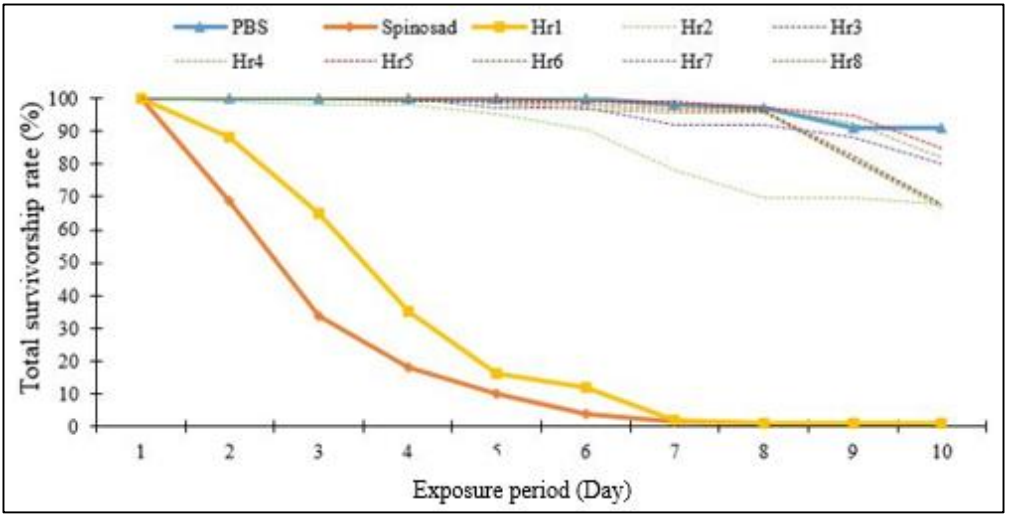

Fig. 1. Pathogenicity of the bacterial isolates (Hr1 to Hr8) compared to positive (Spinosad) and negative (PBS: Phosphate Buffered Saline) controls for an exposure period of 10 days on three replicates of ten healthy third-instar larvae of *Helicoverpa armigera* per treatment each repeated 4 times; data of the percentage of total survival rate are transformed (Templeton 2011).

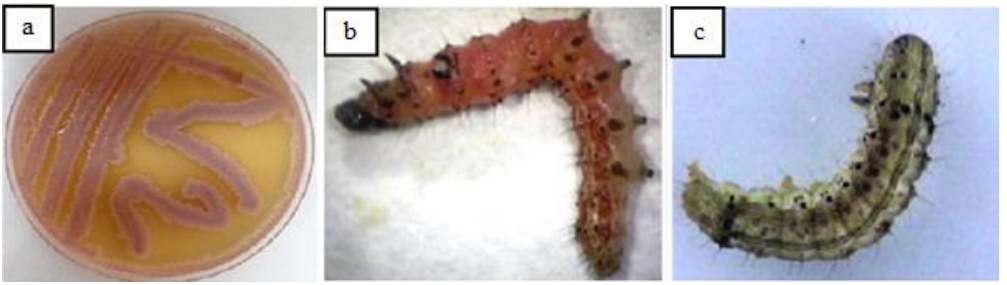

Fig. 2. Aspect of strain Hr1 colonies on solid LB medium (a), effect of Hr1 inoculation on third-instar *Helicoverpa armigera* larvae (b) compared to healthy larvae (c).

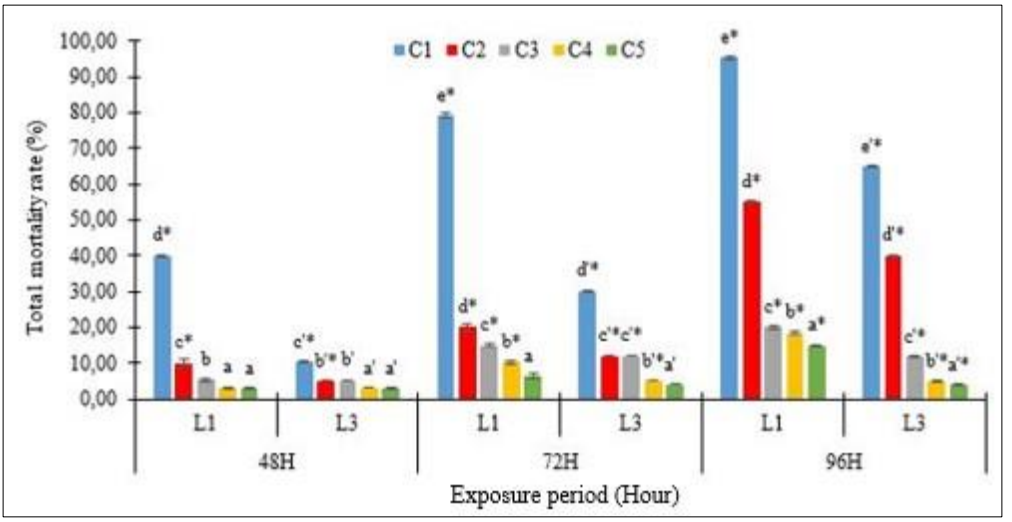

Fig. 3. Dose-response test of the bacterial isolate Hr1 (C1: 1.8×10^9 CFU/ml, C2: 1.8×10^8 CFU/ml, C3: 1.8×10^7 CFU/ml, C4: 1.8×10^6 CFU/ml, C5: 1.8×10^5 CFU/ ml) for an exposure period of 96 h of two batches of three replicates of ten healthy *Helicoverpa armigera* first (L1) and third (L3) instar larvae per treatment repeated 4 times. The different lowercase letters represent statistically significant differences among mortality rates produced by the different concentrations within the larval stage according to SNK multiple comparison tests ($P < 0.05$). Asterisk (*) represents statistically significant differences among mortality rates produced by the different concentrations between the larval stages according to the Student test for independent sample. Vertical bar represents standard deviations. The data of the percentage of total mortality are transformed (Templeton 2011).

Further characterization of Hr1.

The bacterial isolate Hr1 was further characterized by a polyphasic approach. In agreement with 16S rRNA sequencing and using a Biolog identification system equipped with GN III microplates, Hr1 was found to belong to the *Bacillus cereus*/*thuringiensis* clade (probability 96.7 %, SIM 0.682, and DIST 4.531) (Table 3).

The phylogenetic analysis of the seven genes listed in Tourasse-Helgason MLSA scheme revealed that strain Hr1 belonged to cluster IV of the *Bacillus cereus* group (Fig. 4).

The seven strains belonging to the subtree containing bacterial isolate Hr1 (Fig. 5) had all ANI and dDDH values slightly below the species boundary cutoffs compared to *B. thuringiensis* ATCC 10792 (ANI 96.31 – 96.49, dDDH = 64.1 – 69.5) and clearly below the species boundary cutoffs compared to *B. cereus* ATCC 14579(ANI = 95.86 – 96.4, dDDH 65.3– 65.8) Hr1 is not a *B. cereus* but a *B. thuringiensis*. Moreover, all ANI and dDDH values were greater than species boundary cutoffs compared to the type strain *B. cereus* NCTC6474 considered to be *B. thuringiensis* gv. *cytolyticus* (ANI 98.8 – 99.56, dDDH = 77.2 – 88.7). According to these results, Hr1 belongs to the *cytolyticus* subspecies (Table 4).

Table 3. Carbon-source metabolic and chemical sensitivity characteristics of Hr1 in MS 1030 GEN III ID microplates (+: positive response, −: negative response, /: moderate response)

Polymers		**Methyl esters**		**Amino acids, peptides and related chemicals**	
Dextrin	+	Methyl pyruvate	-	D-Aspartic acid	-
Gelatin	+	D-Lactic acid methyl ester	-	L-Arginine	-
Pectin	+	**Carboxylic acids**		γ-Aminobutyric acid	-
Tween 40	-	Acetic acid	-	**Nucleosides**	
Sugars and sugar derivatives		Acetoacetic acid	+	Inosine	+
N-Acetyl-D-galactosamine	-	Citric acid	-	**Alcohols**	
N-Acetyl-D-glucosamine	+	Formic acid	+	Glycerol	+
N-Acetyl-β-D-mannosamine	-	L-Galactonic acid lactone	-	D-Glucose-6-phosphate	+
D-Arabitol	-	D-Galacturonic acid	-	D-Fructose-6-phosphate	+
D-Cellobiose	+	D-Gluconic acid	-	**Else**	
D-Fructose	+	D-Malic acid	-	pH 5	-
D-Fucose	-	L-Malic acid	+	pH 6	+
D-Galactose	-	D-Glucuronic acid	-	1% NaCl	+
Gentiobiose	-	α-Hydroxy-butyric acid	-	4% NaCl	+
α-D-Glucose	+	β-Hydroxy-butyric acid	-	8% NaCl	+
D-Salicin	-	ρ -Hydroxy-phenylacetic acid	-	1% sodium lactate	+
D-Maltose	+	D-Saccharic acid	-	Fusidic acid	-
D-Mannitol	-	Mucic acid	-	Troleandomycin	-
D-Mannose	-	α -Keto butyric acid	-	Rifamycin SV	-
D-Melibiose	-	α -Ketoglutaric acid	-	Minocycline	-
β-Methyl-glucoside	-	Bromosuccinic acid	-	Lincomycin	-
Stachyose	-	**Amino acids, peptides and related chemicals**		Guanidine HCl	+
D-Raffinose	-	L-Alanine	-	Niaproof 4	-
L-Rhamnose	-	L-Aspartic acid	-	Nalidixic acid	-
D-Sorbitol	-	L-Glutamic acid	-	Lithium chloride	+
Sucrose	+	L-Histidine	-	Potassium tellurite	+
D-Trehalose	+	Glycyl-L-proline	-	Aztreonam	+
D-Turanose	-	L-Pyroglutamic acid	-	Sodium butyrate	/
		L-Serine	+	Sodium bromate	+

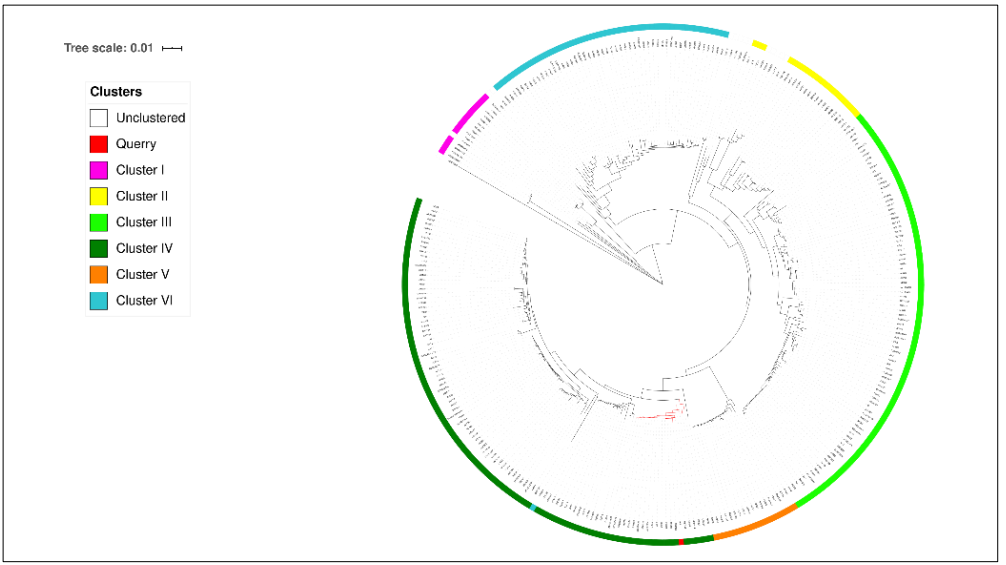

Fig. 4. Neighbour-joining supertree of isolate Hr1 and 315 bacterial strains belonging to the *Bacillus cereus* group based on sequencing of the seven chromosomal genes listed in Tourasse-Helgason scheme (Tourasse et al. 2006). The subtree colored in red contains bacterial isolate Hr1.

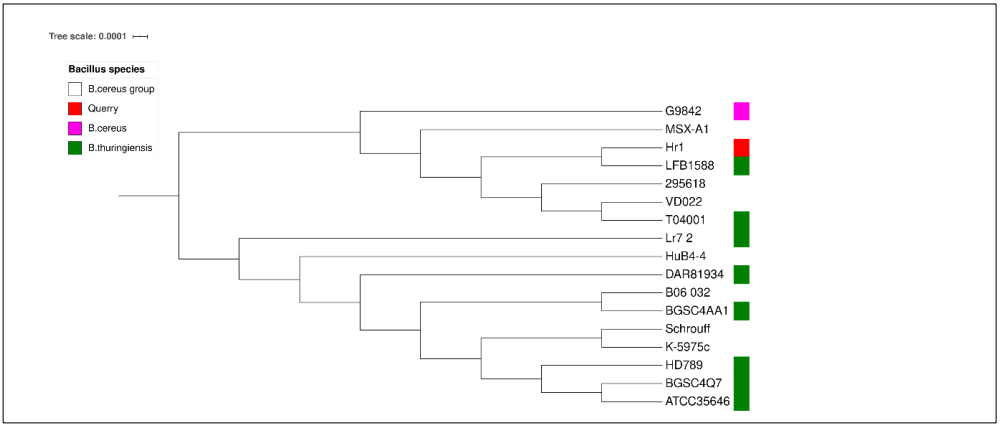

Fig. 5. Neighbour-joining subtree of strain Hr1 and the closest 16 bacterial strains based on sequencing of the seven chromosomal genes listed in Tourasse-Helgason scheme.

Table 4. Pairwise ANI **(**values at the bottom left) and dDDH (values at the top right) values of seven strains close to bacterial isolate Hr1 and 3 reference strains (1: ATCC 14579 (*B. cereus sensulato* type strain) (Baek et al. 2019; Liu et al. 2015), 2: ATCC 10792 (*B. thuringiensis* gv. *berliner* type strain) (Baek et al. 2019; Liu et al. 2015), 3: NCCTC 6474 (*B. thuringiensis* gv. *cytolyticus* type strain) (Baek et al. 2019), 4: G9842, 5: T04001, 6: MSX-A1, 7: VD022, 8: Schrouff, 9: Lr7/2 and 10: k-5975C)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1		71.2	66	65.6	65.8	65.7	65.3	65.6	65.7	65.5
2	96,71		69.1	69.5	68.4	68.7	68.9	68.3	64.1	68.3
3	95,92	96.25		88.7	80.2	78.7	77.2	86.1	78.7	86
4	95,89	96.49	99.01		82.4	84.3	76.9	82.7	88.1	82.6
5	95,88	96.37	98.85	98.96		74.7	75.7	75.6	72.6	75.5
6	95,89	96.31	98.8	99.19	98.69		70.6	73.6	75.5	73.5
7	96,4	96.4	99.13	99.06	99.02	98.81		78.8	73.2	78.8
8	95,94	96.33	99.56	98.95	99.02	98.7	98.92		71.9	99.8
9	95.86	96.48	99.02	99.42	98.99	99.21	99.1	98.93		71.9
10	95.94	96.32	99.55	98.93	98.93	98.72	98.95	99.88	98.94	

*dDDH: Digital DNA-DNA Hybridization. *ANI: Average Nucleotide Identity.

DISCUSSION

Efficiency of Hr1.

The bacterial isolate Hr1 was the most virulent of all other seven isolates against *H. armigera* and had a significant insecticidal effect during both screening and dose-response tests on different larval stages of the pest. Bioassays were conducted on third-instar healthy larvae selected randomly. The percentages of larval mortality observed with the Hr1 isolate were close to those mentioned in the literature. Indeed, Majeed et al. (2018) reported data close to ours by testing the insecticidal effect of a *B. thuringiensis* isolate from Pakistan against *H. armigera* L3 larvae. A 1×10^7 CFU/ml bacterial suspension from this isolate caused 20% larval mortality within two days of initial infection and 65% within four days from initial infection. In the literature, a 1×10^9 CFU/ml bacterial suspension of an Indian isolate of *B. thuringiensis* caused 30% mortality of first-instar *H. armigera* larvae after two days of initial infection and 87% after four days of initial infection (Patel et al. 2018). The dose-response test we conducted with a 1.8×10^9 CFU/ml inoculum of Hr1 showed that Hr1 was the most effective against *H. armigera* first and third-instar larvae. Independently of larval stages, insect larvae are often sensitive to pathogens and more particularly to bacteria (Ravensberg 2011). The effectiveness of a biopesticide is believed to depend mostly on the different developmental stages of the insect (Khetan 2001). For instance, first-instar *Danaus plexippus* larvae (Lepidoptera, Danainae) were 12 to 23 times more sensitive to *B. thuringiensis* toxins than older larvae (Hellmich et al. 2001). Similarly, in our study, juvenile stages were more sensitive to infection with Hr1 than older stages. Indeed, for both concentrations C1 and C2, the percentage of mortality was greater

when larvae were inoculated at the L1 stage. C1 was further tested on the pod borer first instars, then a bacterial suspension of Hr1 was incorporated into a talc and carboxymethyl cellulose (CMC) mixture to get a formulation with a concentration of 1.7×10^9 CFU/g of CMC-talc powder. Tomato plants grown in a greenhouse were infested with the pod borer and treated with the formulated bacterium both with and without rain simulation. Formulated Hr1 remained effective even after rain-wash (Bouslama et al. 2020).

Identification of Hr1.

Partial sequencing of the 16S rRNA gene and the Biolog test revealed that Hr1 belongs to the species *B. cereus* or *B. thuringiensis*. Moreover, the phylogenetic analysis of the seven genes listed in the Tourasse-Helgason MLSA scheme revealed that strain Hr1 belonged to cluster IV of the bacteria from the *Bacillus cereus* group. Species in this clade have high DNA similarity values which makes it almost impossible to identify subspecies (Böhm et al. 2015; Okinaka and Keim 2016). Liu et al. (2015) proposed another clustering system based on Genome BLAST Distance Phylogeny (GBDP) and compared it to an MLSA approach that gave almost the same results. Bacterial isolate Hr1 is phylogenetically very close to seven bacteria whose genomes are fully sequenced. These bacteria all have dDDH values slightly below the species boundary cutoffs as compared to *B. thuringiensis* ATCC 10792 representing cluster BCG04 and clearly below the species boundary cutoffs as compared to *B. cereus* ATCC 14579 representing cluster BCG03. Our results are close to those of Liu et al. (2015), claiming that BCG03, BCG04 and BCG17 are three sharply related clusters sharing a common ancestor and forming a clade within the phylogenetic tree of the *B. cereus* group. Strains BCG17 had dDDH values plainly under 70% compared to BCG03 strains. Nonetheless, they had dDDH values non-significantly lower than 70% with BCG04 strains. Therefore, we can conclude that Hr1 and its seven close strains belong to cluster BGC17. Baek et al. (2019) also recently proposed a new classification for *B. cereus* strains based on genomic relatedness and characteristics of several *B. thuringiensis* and *B. cereus* strains. They proposed to classify these strains into three distinct sub-species, namely *B. cereus* gv. *sensulato B. thuringiensis* gv. *thuringiensis*, and *B. thuringiensis* gv. *cytolyticus*. Strains belonging to the subtree containing bacterial isolate Hr1 had all ANI and dDDH values higher than the species boundary cutoffs compared to the type of strain *B. thuringiensis* gv. *cytolyticus*. Therefore, Hr1 and its seven close strains were identified as *B. thuringiensis* gv. *cytolyticus* strains.

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RESUME

Bouslama T., Laarif A., Chattaoui M., Vial L., Lavire C. et Doré J. et Rhouma A. 2023. Identification biochimique et moléculaire d'un isolat autochtone de *Bacillus thuringiensis* gv. *cytolyticus* ayant un effet insecticide contre les larves de la noctuelle de la tomate (*Helicoverpa armigera*). Tunisian Journal of Plant Protection 18 (2): 71-91.

Le but de cette étude est d'isoler et d'identifier des souches bactériennes autochtones à effet insecticide contre les larves de la noctuelle de la tomate *Helicoverpa armigera*. Des larves mortes et malades ont été collectées dans des parcelles de piment de la région de Gotraniya du gouvernorat de Kairouan en Tunisie. Ces larves ont été utilisées pour isoler huit souches bactériennes dénommées Hr1 à Hr8. Les isolats bactériens ont été caractérisés par des observations macroscopiques et microscopiques et par séquençage de l'ARNr 16S. Hr1, Hr2, Hr4, Hr5, Hr6 et Hr8 ont été identifiés comme *Bacillus* spp., Hr3 comme *Staphylococcus* sp. et Hr7 comme *Enterobacter* sp. Leurs potentiels insecticides ont été évalués contre les larves de troisième stade de *H. armigera*. Un isolat bactérien Hr1 a montré un effet insecticide intéressant contre les larves de *H. armigera*, provoquant une mortalité larvaire de 60% 4 jours après le traitement à une dose de $1,8 \times 10^9$ UFC/ml. Sur la base d'études de caractérisation plus approfondies, Hr1 a été identifié comme étant *Bacillus thuringiensis* gv. *cytolyticus* à la suite d'un test biochimique Biolog et d'analyses de séquences multi-locus basées sur le séquençage de sept gènes conservés.

Mots clés: Analyse de séquence multi-locus, *Bacillus thuringiensis* gv. *cytolyticus*, bactérie entomopathogène, Biolog, lutte biologique, *Helicoverpa armigera*, séquençage de l'ARNr 16S

ملخص

بوسلامة، ثامر وأسماء العريف وميساء الشتاوي ولودفيك فيال وسيلين لافير وجان دوري وعلي رحومة. 2023. تشخيص بيوكيميائي وجزيئي لعزلة بكتيرية محلية من نوع *Bacillus thuringiensis* gv. *cytolyticus* ذات تأثير قاتل على يرقات الفراشة الليلية الطماطم (*Helicoverpa armigera*).

Tunisian Journal of Plant Protection 18 (2): 71-91.

الهدف من هذه الدراسة هو عزل وتشخيص سلالات بكتيرية محلية لها قاتل تأثير على يرقات الفراشة الليلية للطماطم *Helicoverpa armigera*. تم جمع اليرقات الميتة والمريضة من حقول فلفل بمنطقة القطرانية بولاية القيروان في تونس. وتم استخدام هذه اليرقات لعزل ثماني سلالات بكتيرية سُميت من Hr1 إلى Hr8. تم توصيف البكتيريات المعزولة من خلال المشاهدات البصرية والمجهرية وكذلك تسلسل الحمض النووي الريبي الريبوسومي S16. تم تشخيص Hr1 و Hr2 و Hr4 و Hr5 و Hr6 و Hr8 على أنها *Bacillus* spp.، و Hr3 على أنها sp. *Staphylococcus* و Hr7 على أنها sp. *Enterobacter*. وتم تقييم فعالية هذه البكتيريات ضد الطور الثالث من يرقات الفراشة الليلية للطماطم. أظهرت العزلة البكتيرية Hr1 فاعلية مهمة، حيث تسببت في وفاة 60% من اليرقات بعد 4 أيام من العلاج بجرعة 1.8×10^9 خلايا بكتيرية/مل. بناءً على المزيد من الدراسات التوصيفية المعمقة، تم تشخيص Hr1 على أنها *Bacillus thuringiensis* gv. *cytolyticus* بعد إجراء اختبار بيولوجي كيميائي Biolog وتحليل تسلسلي للجينات متعدد المواقع استنادًا إلى تسلسل سبعة جينات محفوظة.

كلمات مفتاحية: بكتيريا مسببة للأمراض الحشرية، تحليل تسلسلي للجينات متعدد المواقع، تسلسل الحمض النووي الريبي الريبوسومي S16، مكافحة بيولوجية، *Bacillus thuringiensis* gv. *cytolyticus*، Biolog، *Helicoverpa armigera*

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APPENDIX

Table A1. List of the primers used in the MLSA scheme

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
adk*	GGT AAA GGT ACA CAA GCC GAA CAG	CTA AGC CTC CGA TGA GAA CAT CG
glpT*	ATG GGC TGG TAT TCC AGG TAC AC	AAG TAA GAG CAA GGA AGA ACA TTG CA
glpF‡	TTG TTA ATC GTA CTT GGT GGC GG	ACT GGA ATC CAT GCA TAC TTC CAG TT
panC#	CGA TAT CCT CGT GAT ATT GAT AGA G	TCC GCA TAA TCT ACA GTG CCT TTC
pycA‡	CTA TGC GTT AGG TGG AAA CGA AAG AC	GTC GGT GTA TCA AGC ACA GAT ACA T
ccpA*	TAT GAT GTA GCG CGT GAA GC	CCT TGT AAC TTC TTC GCG CTT CC
pta‡	CGC TGC AGA GCG TTT AGC AAA AGA A	CTC AGC TAC AGA TGG TAC GAA TGC

*Primers for the *adk*, *glpT* and *ccpA* genes from Helgason et al. (2004).
‡Primers for the *glpF*, *pycA* and *pta*genes from Priest et al. (2004).
#Primers for the *panC* gene from Candelon et al. (2004) and Sorokin et al. (2006).

Table A2. Analysis of variance of the bacterial pathogenicity test

		Sum of squares	DF	Mean Square	F	*P*
Day2	Intergroup	11117.668	9	1235.296	4322.649	< 0.01
	Intragroup	31.435	110	0.286		
	Total	11149.103	119			
Day3	Intergroup	50930.978	9	5658.998	36124.806	< 0.01
	Intragroup	16.918	108	0.157		
	Total	50947.896	117			
Day4	Intergroup	96981.880	9	10775.764	93821.218	< 0.01
	Intragroup	12.404	108	0.115		
	Total	96994.284	117			
Day5	Intergroup	131543.427	9	14615.936	73682.181	< 0.01
	Intragroup	21.225	107	0.198		
	Total	131564.652	116			
Day6	Intergroup	149173.748	9	16574.861	169321.694	< 0.01
	Intragroup	10.572	108	0.098		
	Total	149184.321	117			
Day7	Intergroup	168367.176	9	18707.464	99591.825	< 0.01
	Intragroup	20.099	107	0.188		
	Total	168387.275	116			
Day8	Intergroup	167031.454	9	18559.050	37721.857	< 0.01
	Intragroup	52.152	106	0.492		
	Total	167083.606	115			
Day9	Intergroup	141430.091	9	15714.455	45071.812	< 0.01
	Intragroup	38.003	109	0.349		
	Total	141468.094	118			
Day10	Intergroup	114003.787	9	12667.087	30097.040	< 0.01
	Intragroup	44.192	105	0.421		
	Total	114047.978	114			

*$P < 0.001$ (highly significant); two-way factorial ANOVA at $\alpha = 0.05$.
*DF: degrees of freedom.
*F: Fisher test.
*P: *P*-value.

Table A3. Analysis of variance of the data of the dose-response test on first- and third-instar larvae

			Sum of squares	DF	Mean Square	F	*P*
L1	**48H**	Intergroup	11804.68	4	2951.17	899.62	< 0.01
		Intragroup	173.86	53	3.28		
		Total	11978.54	57			
	72H	Intergroup	40097.46	4	10024.37	1844.20	< 0.01
		Intragroup	277.22	51	5.44		
		Total	40374.68	55			
	96H	Intergroup	55962.28	4	13990.57	7796.75	< 0.01
		Intragroup	93.31	52	1.79		
		Total	56055.59	56			
L3	**48H**	Intergroup	11804.68	4	2951.17	899.62	< 0.01
		Intragroup	173.86	53	3.28		
		Total	11978.54	57			
	72H	Intergroup	40097.46	4	10024.37	1844.20	< 0.01
		Intragroup	277.22	51	5.44		
		Total	40374.68	55			
	96H	Intergroup	55962.28	4	13990.57	7796.75	< 0.01
		Intragroup	93.31	52	1.79		
		Total	56055.59	56			

*$P < 0.001$ (highly significant); two-way factorial ANOVA at $\alpha = 0.05$.
*DF: degrees of freedom.
*F: Fisher test.
*P: p-value.

Table A4. Comparison between mortalities of first- and third-instar larvae associated to different doses of the bacterial strain Hr1

Dose	Time	F	*t*	DF	*P* (2-tailed)	Mean difference	Standard deviation	95% confidence interval	
								Inferior	Superior
	48H	0.194	82.387	22	$P < 0.001$	29.638	0.360	28.892	30.384
C1	**72H**	16.237	57.538	21	$P < 0.001$	48.902	0.850	47.135	50.670
	96H	17.091	51.277	21	$P < 0.001$	30.267	0.590	29.039	31.494
	48H	21.852	4.993	21	$P < 0.001$	4.948	0.991	2.887	7.008
C2	**72H**	22.636	9.199	20	$P < 0.001$	7.952	0.864	6.149	9.755
	96H	7.171	39.381	20	$P < 0.001$	14.988	0.381	14.194	15.782
	48H	19.478	0.225	21	$P > 0.05$	0.109	0.485	-0.900	1.119
C3	**72H**	12.181	5.229	20	$P < 0.001$	2.986	0.571	1.795	4.178
	96H	6.234	20.087	20	$P < 0.001$	8.165	0.406	7.318	9.013
	48H	0.685	0.372	22	$P > 0.05$	0.063	0.168	-0.286	0.411
C4	**72H**	16.884	13.989	21	$P < 0.001$	5.141	0.367	4.377	5.905
	96H	10.914	35.713	21	$P < 0.001$	13.454	0.377	12.671	14.238
	48H	15.747	-0.128	21	$P > 0.05$	-0.050	0.394	-0.870	0.769
C5	**72H**	20.100	3.375	22	$P < 0.05$	2.425	0.718	0.935	3.915
	96H	5.559	39.106	20	$P < 0.001$	10.916	0.279	10.334	11.499

*$P < 0.001$ (highly significant); Student's t-test for independent samples at $\alpha = 0.05$.
*$P < 0.05$ (significant); Student's t-test for independent samples at $\alpha = 0.05$.
*$P > 0.05$ (not significant); Student's t-test for independent samples at $\alpha = 0.05$.
*For $\alpha = 0.05$, t (tabulated) = 1.734 < t (calculated); the average values of the samples were unequal.
*DF: degrees of freedom.
* F: Fisher test.
* t: Student test value.
*P: *P*-value.
