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Isolation, characterization, and bio-insecticidal efficiency of Ethiopian isolates of *Bacillus thuringiensis* against *Galleria mellonella* L. (Lepidoptera: Pyralidae) and tomato whitefly, *Bemisia tabaci* (Genn.) (Hemiptera: Aleyrodidae)

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Abstract

Background: *Bacillus thuringiensis* is a Gram-positive, rod-shaped, and spore-forming microbial entomopathogen. The silverleaf whitefly, *Bemisia tabaci* (Genn.) (Hemiptera: Aleyrodidae), is a worldwide important cryptic species causing serious economic damage to several vegetable crops including tomato. The aim of this study was to characterize and evaluate the bio-insecticidal effects of locally isolated *Bacillus thuringiensis* (*Bt*) against *Galleria mellonella* and *Bemisia tabaci*.

Results: Thirty-one isolates of *Bt* were obtained from 70 soil samples based on cultural characterization coupled with insecticidal crystal protein detection. All of the 31 bacterial isolates were pre-screened for their bio-insecticidal property against the greater wax moth, *Galleria mellonella* L. larvae. Only 20 (64.5%) isolates were virulent to *G. mellonella* with a percentage mortality that ranged from 2.50 to 95%. The *groEL* gene sequences of all 20 entomopathogenic indigenous isolates displayed 99–100% similarity with *Bt* isolates. Bioassay evaluation of 12 selected isolates against 3rd to 4th nymphal instars of *B. tabaci* with spore-crystal suspensions of 1×10^9 spores/ml caused 0 to 31.25%, 0 to 57%, and 0 to 82.5% percentage mortality within 48, 96, and 144 h accordingly. Among isolates of *Bt*, isolate AAUDS-16 had the highest virulence, followed by the isolate AAUES-69D with LC_{50} values of 9.67×10^6 spores/ml and 1.16×10^7 spores/ml, respectively. The virulent isolates were tested for their growth response to a different temperature range between 15 and 40 °C. All isolates showed a maximum growth rate around 30 °C.

Conclusions: *Bt* isolates of AAUES-69D and AAUDS-16 displayed high insecticidal potential against *B. tabaci* nymphs and *G. mellonella* larvae, and showed a maximum growth rate in a wide range of temperature. High virulence and temperature-tolerant isolates should be important candidates for diverse insecticidal toxin studies.

Keywords: *Bacillus thuringiensis*, Crystal inclusion, Bio-assay evaluation, *Bemisia tabaci*, *Galleria mellonella*

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Background

In Ethiopia, tomato (*Lycopersicon esculentum* Mill.) is an important food ingredient in a daily diet, a source of cash-generating to small-scale farmers, and delivers employment in both production and processing industries. Its production is mainly challenged by numerous abiotic and biotic factors (Sora 2018). In Ethiopia, insect pests (Ayalew et al. 2009) and associated diseases (Mandefro et al. 2009) are the most important biotic factors contributing to the lowest yield of tomatoes.

The silver leaf whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), is a global important cryptic species complex, resulting in serious economic damage on tomatoes because of its direct and indirect destruction (Shelby et al. 2020). The wide use of chemical pesticides poses a great threat to the environment and food safety (Sparks and Nauen 2015). The health hazards, environmental problems, and insect resistance associated with excessive use of chemicals necessitate for search-integrated pest management strategy (Aramideh et al. 2010).

Microbial biopesticides are used as a biocontrol agent against a wide variety of agricultural insect pests in many agroecosystems (Ruiu 2018). These microbes are natural enemies of insects, target-specific, and promising in reducing the use of hazardous chemical pesticides (Majeed et al. 2017). Among microbial entomopathogens, *Bacillus thuringiensis* (*Bt*) is the most important spore-forming microbial bio-control agent, which produces insecticidal crystal (*Cry*) and cytolytic (*Cyt*) protein (δ -endotoxins) encoded by *cry* and *cyt* genes (Soberon et al. 2018) and *Vip* protein (Chakroun et al. 2016). The insecticidal proteins are solubilized in the insect intestine after ingested, freeing protoxins that bind to specific receptors in the intestinal epithelium causing pore formation on the cellular membrane, which leads to an ionic imbalance and larval death (Soberon et al. 2018). The bacterium is the most successfully commercialized bio-control agent worldwide for its high specificity and associated environmental safety (Jurat-Fuentes and Crickmore 2017). However, excessive application of *Bt* has resulted in reduced efficacy as a result of resistance development with insect pests (Zago et al. 2014). This limited pathogenicity efficacy resolved with isolation and identification of propitious indigenous isolates. Hence, isolation and identification of new *Bt* strains from soil environment with privileged insecticidal properties is a vigorous research priority in many regions of the world (Reyaz et al. 2017).

Native isolates of *Bt* from soil environments caused up to 50 to 70% mortality of *B. tabaci* nymphs (Cabra and Hernandez Fernandez 2019) and significantly reduced *G. mellonella* within 48 h post-infection (Grizanova et al. 2019). In Ethiopia, Woldetenssay and Ashenafi (2008) studied the distribution of *Bt* on different agroecological

soils of Ethiopia and found that 32% of the tested 503 soil samples harbored *Bt*. They showed that 44 (21%) isolates killed 50–100% of *Anopheles arabiensis* larvae within 48 h.

Therefore, there is a great potential to look for isolation and characterization of new *Bt* strains to find out strains with novel or high insecticidal activities. In this context, the present study was initiated to characterize the *Bt* isolates from soil samples of Menagesha forest and Rift-valley farmlands and evaluate their insecticidal activity against *B. tabaci* and *G. mellonella* under in vitro conditions.

Methods

Collection of soil samples

A total of seventy (70) soil samples were collected from Dalota, Kality, Erobgebaya, Koka, and Menagesha forest sites, Ethiopia. Samples were collected by scraping off the surface soil, and from each site, approximately 1.5 kg of soil samples were collected from 2 to 5-cm depth and placed in alcohol-sterilized polyethylene plastic bags. Collected soil samples were brought to the Applied Microbiology laboratory and stored at 4 °C for further processing.

Isolation of entomopathogenic bacterium *Bt* from soil

A modified version of the temperature selection method was used to isolate *Bt* from soil samples as described by Travers et al. (1987). Soil samples (10 g) were individually mixed in 100 ml sterile water and homogenized with an orbital shaker (200 rpm) for 4 h at room temperature. After complete homogenization, the samples were prepared to appropriate dilution from which samples were taken and heated at 80 °C for 10 min in a water bath equipped with a shaker to destroy non-spore formers and vegetative *Bacillus* cells. Then, 0.1 ml soil of aliquot samples was individually inoculated into nutrient agar and incubated at 28 °C for 48–72 h. Colonies were randomly picked and subcultured on nutrient agar and maintained for further investigation.

Cultural characterization

A total of 280 bacillus colonies were picked and characterized for their cultural characteristics such as colony's color, shape, margin, elevation, and surface. Bacterial colonies showing typical *Bt* were preliminary identified based on cultural and morphological characteristics regarding *Bergey's Manual of Determinative Bacteriology* (Halt et al. 1994).

Morphological characterization

Gram staining

Gram staining of isolates was carried out following the protocol of Provine and Gardner (1974). Thin bacterial

smear was made on a clean glass slide, dried in the air, and heat-fixed. The smear was covered with crystal violet and kept for 1 min. The slide was washed, covered with Gram's iodine, and let stand for 1 min. The slide was washed, decolorized with acetone, rocking the slide gently. The decolorization step was done very quickly. Immediately, the slide was washed with water, counter-stained with safranin and let stand for 30 s, washed with water, blot dried, and examined under the microscope ($\times 100$ magnifications).

Coomassie brilliant blue staining

The presence of parasporal bodies of isolates was detected, using Coomassie brilliant blue (CBB) staining, following the protocol of Rampersad et al. (2002). Selected isolates were inoculated into a sterile 50-ml conical flask containing nutrient broth and incubated in an orbital shaker (250 rpm) for 90 to 110 h at room temperature. Samples were smeared onto glass slides, followed by an air dry and heat fixing. Then, the slides were stained using 0.133% Coomassie blue stain in 50% acetic acid for 5 min. The dried slides were then observed under a phase-contrast microscope ($\times 100$) to visualize the formation of parasporal bodies and spores.

Preparation of bacterial suspension

Isolates from nutrient agar slants were transferred into the fresh nutrient broth and incubated at 28 °C aerobically for complete sporulation and lysis of all vegetative cells. Completion of sporulation and lysis (spore-crystal formation) was detected by using a hemocytometer with the aid of a phase-contrast optical microscope (Soares-da-Silva et al. 2015). The spore-crystal mixture of each isolate was standardized at a concentration of 1×10^9 spores/ml for bio-assay.

Preliminary pathogenicity screening of bacterial isolates using *G. mellonella* under laboratory condition

Rearing of *Galleria mellonella*

Rearing of larvae of *Galleria mellonella* L. (Lepidoptera, Pyralidae) was undertaken according to Meyling (2007) at the Ambo Plant Protection Agricultural Research Center. Adult moths were kept in 500-ml flasks containing folded tissue paper to facilitate their mating and egg-laying potential. Eggs were laid on folded tissue paper, and each tissue paper was transferred from a flask into rearing plastic containers containing honey, wheat bran, and glycerol as dietary components for hatching larvae. The plastic containers were incubated at 20 °C for 4 weeks under darkness. The resulting third to fourth instar larvae were used for bio-assay evaluation.

Bio-assay evaluation

Insecticidal activities of isolates were preliminarily bio-assayed on the *G. mellonella* larvae, using the diet contamination technique (Navon et al. 1990). From each culture, 5 ml of spore-crystal suspension containing 1×10^9 spores/ml prepared and mixed with 15 g artificial diet containing honey, wheat bran, and glycerol. Then, 10 larvae of *G. mellonella* were transferred into an artificial diet containing a spore-crystal suspension mixture for feeding. The same number of larvae in an artificial diet without spore-crystal suspension was included as a control. All experiments were repeated 4 times. Periodic mortality was recorded every 48 h for 144 h.

Molecular characterization of entomopathogenic bacterial isolates

DNA extraction

The genomic DNA of isolates was extracted by the heat shock method using TE buffer (Ricieto et al. 2013). Isolates were grown on the nutrient agar at 30 °C for 15 h. Collected 10–20 mg bacterial isolates from the growing colony of approximately 1–2 mm in diameter was transferred to 200 μ l of Tris-EDTA buffer (10 mM Tris; 1 mM EDTA; pH 8.0), using a sterile toothpick. The suspension was homogenized and incubated for 10 min in a boiling water bath (100 °C) for lysis of bacterial cells. Then, the suspension was centrifuged at 10,000g for 5 min to remove debris. The supernatant was transferred to a new microcentrifuge tube and stored at -20 °C for PCR amplification reactions (polymerase chain reaction), and 5 μ l of the supernatant was used as a template in the PCR reaction.

PCR amplification

The amplification of 533 bp groEL gene of *Bt* isolates was carried out, using *B. cereus* group-specific primer BalF (5'-TGCAACTGTATTAGCACAAGCT-3') and BalR (5'-TACCACGAAGTTTGTTCACTACT-3') as described by Chang et al. (2003). Five microliters of the template was amplified in 25 μ l of reaction mixture consisting of 10 mM Tris-HCl (pH-9), 50 mM KCl, 1.5 mM MgCl₂, 200 μ M of each dNTP, 1 U *Taq* DNA polymerase (Fermentas), and 10 pmol of respective primers. The PCRs were performed using a thermocycler (Mastecycler, Eppendorf, Germany) using BalF and BalR primers consist of 30 cycles of 94 °C for 45 s (denaturation), 55 °C for 45 s (annealing), and 72 °C for 45 s (extension). The amplified PCR products were analyzed in 1.5% agarose gel prepared in TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) containing ethidium bromide (0.3 μ g/ml). Electrophoresis was carried out at 7 V/cm for 1 h and 30 min in a submarine gel electrophoresis system (Bangalore Genei, India), and the gel was photographed by UV gel documentation system (Alpha

Innotech Corporation, USA). The PCR product was purified by using the QIAquick PCR purification kit according to the manufacturer's instructions (QIAGEN, Germany) and sequenced. Finally, sequenced data were submitted to NCBI (National Center for Biotechnological Information) and compared to publish sequence of NCBI database for the identification of bacterial isolates at the species level.

In vitro bioassay evaluation of bacterial isolates against *B. tabaci*

Rearing of whiteflies

Whitefly adults were collected from Koka in the tomato field using a manual aspirator. The area is characterized by a mean minimum and maximum temperature of 12.14 °C and 27.39 °C, respectively Ethiopian sugar development agency (ESDA 2010). After collection, insects were reared on young tomatoes planted in pots in whitefly-proof cages (Safavi and Bakhshaei 2017). Tomato seedlings with 2 or 4 leaves were placed into a cage and allowed to be infested by adult whiteflies (sex ratio \approx 1:1). Adults were allowed to oviposit for 24 h on leaves. Then, adults were removed, and plants with whitefly eggs were transferred to other insect-free cages. Whitefly eggs were allowed to develop into the nymphal stage, and 3rd and 4th nymphal instars were applied in bioassays

Single-concentration bioassay

Virulent isolates were evaluated against the nymph of *B. tabaci* under laboratory conditions with the leaf disc method (Ateyyat et al. 2009). The leaf discs with 3rd to 4th nymphal instars were cut from plants and immersed in 1×10^9 spores/ml concentration for 10 s and placed upside down onto 0.2% plain water agar medium in Petri plates. Four replicates (Petri dishes) were set per treatment. Nymphs were considered dead if body color changed from yellowish to dark brown or if the body appeared dry. Periodic mortality was recorded every 48 h for 144 h. Isolates with mortality greater than 50% were selected to estimate the median lethal concentration (LC_{50}).

Multiple-concentration bioassays

The multiple-concentration bioassays were evaluated to estimate LC_{50} , following the leaf disc method as before (Ateyyat et al. 2009). For this study, four (4) highly virulent isolates were selected and evaluated. Leaf discs with 3rd to 4th nymphal instars were immersed in the 5 concentration ranges from 1×10^5 to 1×10^9 spores/ml of each isolate. For each concentration, 4 repetitions were performed, and periodic mortality was recorded every 48 h for 144 h.

Effect of temperature on the growth of isolates

The growth rate response of isolates to different temperatures was evaluated as described by Hamedo (2016) with a little modification. Isolates were inoculated on nutrient broth and incubated for 24 h. One milliliter of each broth culture (cell suspensions) was inoculated into 50 ml sterilized nutrient broth in a 250-ml Erlenmeyer flask and incubated at 15, 20, 25, 30, 35, and 40 °C. The vegetative growth phase of isolates was determined by the growth curve experiment. The maximum growth rate of isolates was measured by taking optical density reading at 600 nm in 6-h intervals starting from 0 h, using a spectrophotometer. Optical density reading and colony-forming units (CFU) were used to plot the regression line, and the maximal growth rate of each isolates in response to different temperatures was calculated.

Data analysis

Mortality data were corrected using Abbot's formula (Abbott, 1925), arcsine transformed, and subjected to the ANOVA procedure of SPSS version 20.0. Means were separated using Tukey's honestly significant difference (HSD) at 5% significance level for screening experiments against *G. mellonella* and *B. tabaci*, respectively. The LC_{50} and LC_{90} values of isolate were determined by using probit analysis (Finney, 1971).

Results

Cultural characterization of bacterial isolates

A total of 280 *Bacillus* isolates were detected using heat treatment methods from 70 soil samples collected from Menagesha forest and various tomato-cultivated land of the central rift valley of Ethiopia. The heat treatment method eliminates non-spore formers and vegetative *Bacillus* cells in the soil samples. All of the 280 *Bacillus*-like colonies were culturally characterized by examining the different characters including colony color, elevation, margin, shape, and colony surface. The cultural study showed that a total of 40 isolates were carefully chosen based on typical colonial characteristics of *Bt* showing white to off white colony color, regular to irregular colony margin, circular colony shape, wrinkled to wavy colony surface, and opaque and flat to slightly raised colony elevation. The result showed that the different isolates showed variations among one another in their cultural characteristics.

Morphological characterization

Gram staining

Culturally characterized isolates were subjected to gram staining to approve the bacterial colonies to be Gram-positive. About 40 isolates selected based on colony

morphology were evaluated for gram staining. Isolates that were rod-shaped and blue indicate Gram-positive isolates. Based on this result, all isolates were found to be Gram-positive, rod-shaped, and spore formers. The isolate absorbed the crystal violet and did not decolorize with the decolorized agent indicating that the cell wall of the organism was composed of peptidoglycan.

Coomassie brilliant blue staining

After Gram staining, isolates were subjected to Coomassie brilliant blue staining as the best method to detect *Bt* isolates from the *Bacillus cereus* group. The phase-contrast microscope analysis revealed that from the total of 40 isolates stained with Coomassie brilliant blue staining, 31 isolates were found to have parasporal inclusion (insecticidal proteins) and classified as *Bt* (Table 1). In contrast, the rest of the 9 isolates were not grouped under *Bt* due to they failed to have parasporal inclusion. Isolates did not have any crystal bodies might be grouped under *Bacillus cereus* or other *B. cereus* group. The parasporal crystalline inclusions of *Bt* isolates were stained as dark blue (Fig. 1). The crystal morphology results of *Bt* isolates were indicated 8 (25.8%) bipyramidal, 5 (16.13%) pyramidal, 7 (25%) spherical, 5 (16.13%) oval, 4 (12.9%) rod, and 2 (6.5%) amorphous crystal shapes. The crystal protein morphology variation of isolates might be due to the genetic variation of entomopathogenic bacterial isolates.

The *Bt* index was calculated as the number of identified *Bt* isolates divided by the total number of *Bacillus*-like colonies examined. Based on the result, the Kality sampling site showed the highest *Bt* index with 0.17,

followed by Koka with 0.13 *Bt* index. Menagesha forest sampling site showed the lowest *Bt* index with 0.09, and an average *Bt* index of the total sampling sites was 0.11 (Table 2).

Preliminary pathogenicity screening of isolates using *G. mellonella* larvae under laboratory condition

Isolates found to have insecticidal proteins were selected and primarily assessed for their toxicity against the *G. mellonella* larvae. The preliminary toxicity screening of 31 isolates with concentrations of spore-crystal suspensions (1×10^9 spores/ml) exhibited a wide range of toxicity toward 3rd instar larvae of *G. mellonella*. From the total of 31 isolates evaluated, 20 (64.5%) isolates showed insecticidal effect, and the mortality of larvae ranged from 2.50 to 62.50%, 10 to 75%, and 20 to 95% within, 48, 96, and 144 h, respectively (Table 3).

The mortality percentage of larvae after 48 h of treatment application ranged between 2.50 and 62.50%. The maximum percentage of mortality was recorded (62.5%) with isolate AAUES-20, whereas the minimum one (2.5%) was recorded by isolate AAUDS-18. Isolate AAUES-20 and AAUMS-11 showed the highest (75%) and lowest (10%) percentages larval mortality after 96 h of treatment accordingly. After 144 h of application of spore-crystal mixture, isolate AAUES-36A caused the maximum mortality (95%), whereas isolate AAUMS-11 triggered the least mortality (20%). There was a discrepancy in toxicity against *G. mellonella* among tested isolates at $P < 0.01$. The result indicated that the percentage of larval mortality increases when larval exposure time of spore-crystal mixture increases (Table 3).

Table 1 The crystal morphology of *Bacillus thuringiensis* (*Bt*) isolates from Ethiopia

No.	Isolates	Crystal formation	Shape of crystal	Isolates	Crystal formation	Shape of crystal
1	AAUDS-4	+	Pyramidal	AAUKoS-12	+	Pyramidal
2	AAUDS-11	+	Bipyramidal	AAUKoS-6	+	Oval
3	AAUDS-16	+	Spherical	AAUMS-11	+	Pyramidal
4	AAUDS-15	+	Spherical	AAUMS-54	+	Bipyramidal
5	AAUDS-17	+	Bipyramidal	AAUMS-50	+	Spherical
6	AAUDS-18	+	Oval	AAUMS-23	+	Pyramidal
7	AAUDS-12	+	Amorphous	AAUMS-22	+	Spherical
8	AAUKS-19	+	Spherical	AAUMS-19	+	Spherical
9	AAUKS-92	+	Bipyramidal	AAUMS-12	+	Amorphous
10	AAUKS-16	+	Spherical	AAUMS-16	+	Rod
11	AAUKS-11	+	Rod	AAUMS-8	+	Oval
12	AAUKS-7	+	Oval	AAUES-6	+	Bipyramidal
13	AAUKS-9	+	Rod	AAUES-36A	+	Pyramidal
14	AAUKS-13	+	Bipyramidal	AAUES-69D	+	Bipyramidal
15	AAUKS-15	+	Oval	AAUES-20	+	Bipyramidal
16	AAUKoS-9	+	Rod			

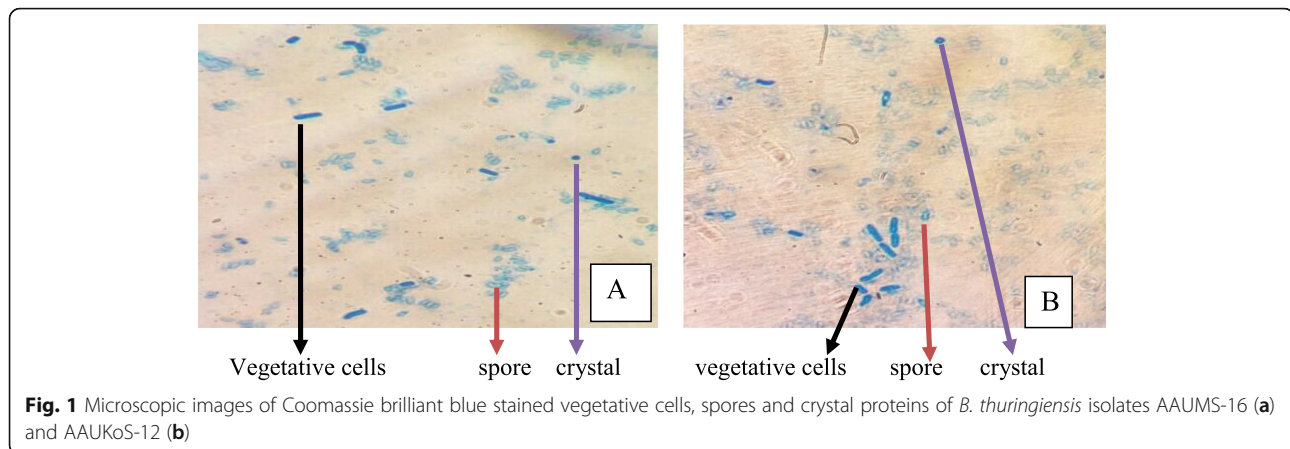


Fig. 1 Microscopic images of Coomassie brilliant blue stained vegetative cells, spores and crystal proteins of *B. thuringiensis* isolates AAUMS-16 (a) and AAUKoS-12 (b)

Molecular characterization of bacterial isolates

A total of 20 isolates of *Bt* screened from cultural characterization, crystal inclusion analysis, and preliminary bioassay evaluation were selected for molecular characterization study. The entomopathogenic bacterial isolates were identified based on groEL gene detection with the heat-shock method. All isolates (100%) produced 533 bp amplified PCR products of groEL genes by using *B. cereus* group-specific primers, BalF, and BalR (Fig. 2 a and b). The sequences of the groEL gene of all indigenous isolates showed 99–100% BLAST search similarity with *Bt* isolates excavated in GeneBank.

The phylogenetic tree of indigenous isolates was constructed by neighbor-joining methods using the mega software (mega4.1) with 1000 bootstrap replications. The neighbor-joining examination showed that isolates of *Bt* from different soil sample sites of Ethiopia were closely related. The bootstrap displayed at the nodal branches and their corresponding accession numbers of isolates were indicated in aside (Fig. 3). Phylogenetic analysis showed that all isolates were positioned as part of the species of *Bt*, and their comparable accession number placed in digression. Isolates of AAUDS-11, AAUKS-15, AAUDS-16, AAUKS-9, AAUKS-92, AAUKS-16, AAUKS-13, AAUES-6, and AAUMS-54 proved to be

very close to each other, and they were all clustered in the *Bt* clade. Besides strains of AAUMS-11, AAUDS-18, AAUES-8, and AAUES-36A were in the same clade even if the genetic distance of these strains in respect to *Bt* AY112850 might be suggested a new variety or subtype.

Bioassay evaluation of *Bt* against *B. tabaci* under in vitro conditions

Isolates showed greater than 80% mortality on *G. mellonella* larvae at 144 h were selected and further screened against whitefly (*B. tabaci*). Bioassay evaluation of twelve (12) isolates of *Bt* against nymphs of *B. tabaci* with a concentration of spore-crystal suspension (1×10^9 spores/ml) caused 0–31.25%, 0–57.5%, and 0–82.5% percentage mortality within 48, 96, and 144 h, respectively (Table 4).

In the 48-h treatment against nymph of *B. tabaci*, the highest mortality was achieved by the isolate AAUES-69D (31.25%), followed by isolate AAUES-20 (27.50%), whereas isolates AAUKS-13 and AAUMS-54 caused the lowest mortality (0%). Isolates of AAUDS-16 and AAUMS-54 caused the maximum (57.5%) and the minimum mortality (0%) against *B. tabaci* nymph after 96-h treatment with spore-crystal mixture respectively. The maximum percentage mortality (82.5%) was recorded by

Table 2 Soil sample collection sites, number of isolates examined, and crystal-forming isolates

No.	Sample sites	No. of soil sample collected	Collection zone	Habitat	Bacillus-like colony examined	Crystal-forming isolates	<i>Bacillus thuringiensis</i> index
1	Dalota	15	East Shoa	Cultivated land	70	7	0.10
2	Kality	10	East Shoa	Cultivated land	47	8	0.17
3	Erobgebaya	15	East Shoa	Cultivated land	38	4	0.11
4	Koka	10	East Shoa	Cultivated land	23	3	0.13
5	Menagesha	20	West Shoa	Forest	102	9	0.09
	Total	70			280	31	0.11

Table 3 Insecticidal activities of *Bacillus thuringiensis* against *Galleria mellonella* larvae at 48, 96, and 144 h

No.	Isolates	% mortality (mean ± SE)		
		48 h	96 h	144 h
1	AAUDS-11	62.50 ± 6.29 ^d	62.50 ± 10.30 ^{de}	80.00 ± 7.07 ^{cdef}
2	AAUDS-16	52.50 ± 2.50 ^{bcd}	60.00 ± 4.08 ^{de}	65.00 ± 5.00 ^{bcd}
3	AAUDS-18	2.50 ± 2.50 ^a	30.00 ± 10.80 ^{abc}	72.50 ± 8.54 ^{bcdef}
4	AAUDS-12	55.00 ± 2.89 ^{cd}	65.00 ± 6.46 ^{de}	70.00 ± 9.13 ^{bcde}
5	AAUKS-19	50.00 ± 4.08 ^{bcd}	47.50 ± 6.29 ^{bcde}	80.00 ± 7.07 ^{cdef}
6	AAUKS-92	40.00 ± 4.08 ^{bc}	55.00 ± 6.45 ^{cde}	82.50 ± 11.81 ^{cdef}
7	AAUKS-16	45.00 ± 6.46 ^{bcd}	62.50 ± 8.53 ^{de}	67.50 ± 7.50 ^{bcd}
8	AAUKS-9	35.00 ± 9.57 ^b	60.00 ± 12.24 ^{de}	92.50 ± 2.50 ^{ef}
9	AAUKS-13	5.00 ± 2.89 ^a	40.00 ± 7.07 ^{bc}	55.00 ± 8.66 ^b
10	AAUKS-15	42.50 ± 7.50 ^{bcd}	62.50 ± 8.53 ^{de}	72.50 ± 4.79 ^{bcdef}
11	AAUKoS-12	35.00 ± 8.66 ^b	70.00 ± 9.12 ^e	87.50 ± 9.46 ^{def}
12	AAUMS-11	5.00 ± 5.00 ^a	10.00 ± 7.07 ^a	20.00 ± 7.07 ^a
13	AAUMS-54	52.50 ± 4.79 ^{bcd}	62.50 ± 2.50 ^{de}	82.50 ± 4.78 ^{cdef}
14	AAUMS-23	12.50 ± 6.29 ^a	27.50 ± 11.82 ^{ab}	50.00 ± 10.80 ^b
15	AAUMS-16	52.50 ± 7.50 ^{bcd}	55.00 ± 6.46 ^{cde}	67.50 ± 4.79 ^{bcd}
16	AAUMS-8	42.50 ± 7.50 ^{bcd}	65.00 ± 10.41 ^{de}	62.50 ± 7.50 ^{bc}
17	AAUES-6	50.00 ± 4.08 ^{bcd}	60.00 ± 10.80 ^{de}	87.50 ± 4.78 ^{def}
18	AAUES-36A	60.00 ± 4.08 ^d	60.00 ± 7.07 ^{de}	95.00 ± 5.00 ^f
19	AAUES-69D	60.00 ± 8.17 ^d	62.50 ± 6.29 ^{de}	82.50 ± 4.79 ^{cdef}
20	AAUES-20	62.50 ± 4.78 ^d	75.00 ± 9.57 ^e	92.50 ± 4.78 ^{ef}
21	Control	-	-	-

Mean with different letters in the column indicates the significant difference at Tukey HSD test, $P < 0.05$

SE standard error, CL confidence limit

the isolate AAUES-69D, and the minimum mortality (0%) was reached by the isolate AAUMS-54 after 144 h of application. The percentage mortality of whitefly infected with *Bt* isolates showed significant variation ($P < 0.01$).

Isolates with mortality greater than 62% against whitefly nymphs were selected to estimate the LC_{50} values. Multiple concentration (1×10^5 – 1×10^9) response

evaluations of the 4 most promising isolates of *Bt* were performed on *B. tabaci* nymphs under laboratory conditions for 144 h. The LC_{50} values of isolates were observed in the range of 9.67×10^6 to 1.21×10^8 spores/ml (Table 5). The maximum LC_{50} value (1.21×10^8 spores/ml) was recorded by isolate AAUMS-23, followed by isolate AAUES-36A (3.20×10^7 spores/ml), whereas the minimum LC_{50} value of 9.67×10^6 spores/ml was indicated by isolate AAUDS-16. The percentage mortality of *B. tabaci* nymphs was recorded after 144 h of post-application of *Bt* isolates in multiple-concentration assessment ranged from 1×10^5 to 1×10^9 spore/ml. The highest mortality was achieved by isolate AAUES-69D at 1×10^5 , 1×10^8 , and 1×10^9 spore/ml, while at the concentrations of 1×10^6 and 1×10^7 spores/ml, the maximum mortality of *B. tabaci* was displayed by isolate AAUDS-16. The percentage mortality of *B. tabaci* was observed to increase with a consequent increase in the concentrations of toxin-spore suspension of selected isolates of *Bt* (Fig. 4). All the treatments exhibited significant differences in percent mortality of *B. tabaci* from different treatments.

Effect of temperature on the growth of *Bt* isolates

The virulent isolates were tested for their growth response to various temperatures (Fig. 5). The mean growth rate of isolates ranged from 0.08 to 0.83, 0.97 to 1.57, 1.77 to 2.73, 2.56 to 3.06, 2.07 to 2.73, and 0.83 to 1.70 generations/h at 15, 20, 25, 30, 35, and 40 °C accordingly. Even though isolates were grown in all tested temperature range from 15 to 40 °C, all of the isolates showed the maximum growth rate (generations/h) around 30 °C. The height growth rate was recorded by an isolate of AAUES-69D (3.06 generations/h), whereas the lowest growth rate was recorded with isolates of AAUMS-23 (2.56 generations/h) at 30 °C.

Average growth rate of isolates in response to all tested temperatures ranged between 1.54 and 2.07 growth rates (generation/h). An isolate of AAUES-69D exhibited the maximum growth rate (2.07 generation/h),

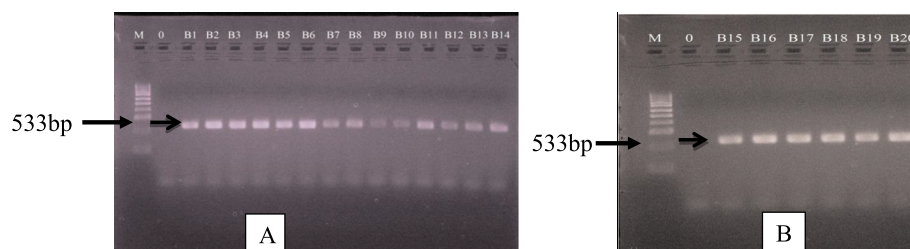


Fig. 2 Agarose gel electrophoresis of amplified PCR products of *groEL* genes using *Bacillus cereus* group specific primer of BalF and BalR from twenty selected isolates of *Bacillus thuringiensis* from Ethiopia **a** and **b**. M, 100 bp ladder; 0, blank; B1, AAUDS-11, B2, AAUKS-15, B3, AAUDS-16, B4, AAUKS-9, B5, AAUKS-92, B6, AAUKS-16, B7, AAUKS-13, B8, AAUES-6, B9, AAUMS-54, B10, AAUMS-11, B11, AAUDS-18, B12, AAUES-8, B13, AAUES-36A, B14, AAUES-69D **a** and M, 100 bp ladder; 0, blank; B15, AAUES-20; B16, AAUMS-23; B17, AAUMS-16; B18, AAUKOS-12; B19, AAUKS-19; B20, AAUDS-12 **b**

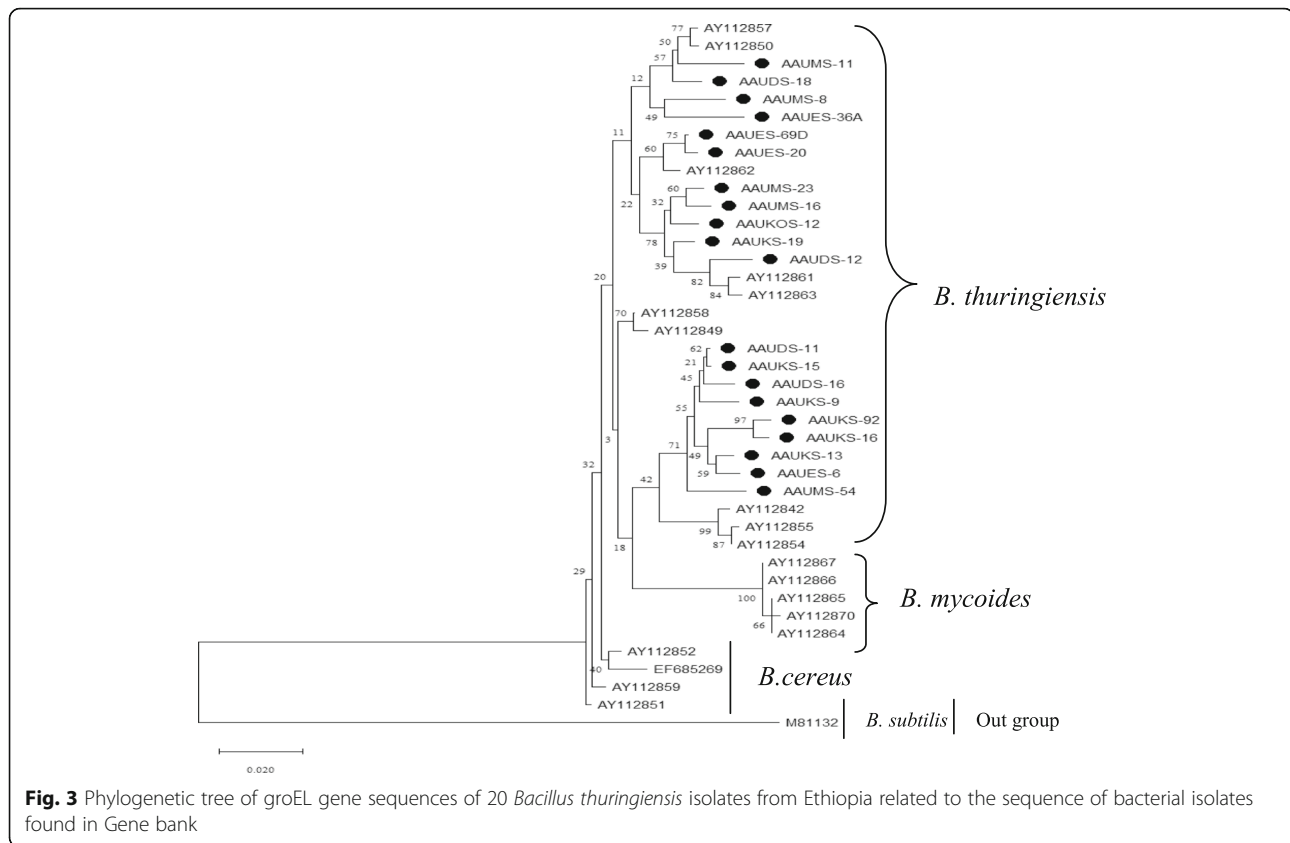


Table 4 Bioassay evaluation of *Bacillus thuringiensis* against whitefly, *Bemisia tabaci*

No.	Isolates	% mortality of <i>Bemisia tabaci</i> (mean ± SE)		
		48 h	96 h	144 h
1	AAUDS-16	21.25 ± 4.27 ^{cdef}	57.50 ± 7.21 ^g	63.75 ± 6.57 ^d
2	AAUDS-18	3.75 ± 2.39 ^{ab}	13.75 ± 4.73 ^{ab}	18.75 ± 5.15 ^b
3	AAUKS-13	0.00 ± 0.00 ^a	21.25 ± 5.15 ^{bc}	40.00 ± 4.56 ^c
4	AAUKS-9	12.50 ± 6.61 ^{abcd}	38.75 ± 2.39 ^{def}	61.25 ± 4.27 ^d
5	AAUKoS-12	25.00 ± 3.54 ^{cdef}	33.75 ± 3.75 ^{cde}	41.25 ± 3.75 ^c
6	AAUMS-23	11.25 ± 4.27 ^{abcd}	43.75 ± 3.75 ^{defg}	72.50 ± 5.20 ^{de}
7	AAUMS-8	10.00 ± 4.56 ^{abc}	28.75 ± 2.39 ^{bcd}	36.25 ± 4.27 ^c
8	AAUMS-54	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 00 ^a
9	AAUES-6	16.25 ± 6.88 ^{bcd}	38.75 ± 4.27 ^{def}	42.50 ± 4.33 ^c
10	AAUES-36A	8.75 ± 3.75 ^{abc}	51.25 ± 7.74 ^{fg}	71.25 ± 2.39 ^{de}
11	AAUES-69D	31.25 ± 4.73 ^f	50.00 ± 6.77 ^{efg}	82.50 ± 4.33 ^e
12	AAUES-20	27.50 ± 5.95 ^{ef}	45.00 ± 6.46 ^{defg}	58.75 ± 7.18 ^d
13	Control	-	-	-

Mean with different letters in the column indicates the significant difference at Tukey HSD test, $P < 0.05$

SE standard error, CL confidence limit

followed by AAUDS-16 (1.86 generation/h), whereas the minimum growth rate was recorded with isolate AAUMS-23 (1.54 generation/h). Generally, isolates of AAUES-69D and AAUDS-16 displayed high insecticidal potential against nymphs of *B. tabaci* with minimum LC₅₀ values of 1.16×10^7 and 9.67×10^6 spores/ml, respectively; also, these isolates showed a maximum growth rate of 2.07 and 1.86 generation/h, respectively, in a response to a wide range of temperature accordingly.

Discussion

In this particular study, cultural, morphological, molecular, and bio-insecticidal activities were used to describe *Bt* isolates from soil samples collected from Menagesha forest and various tomato-cultivated land of the central rift valley of Ethiopia. A total of 280 *Bacillus* isolates were detected from 70 soil samples using heat treatment methods. Likewise, Woldetenssay and Ashenafi (2008) isolated a total of 213 *Bacillus*-like colonies from 503 soil samples collected from the different agro-ecological zone of Ethiopia. Another study showed that about 89 *Bacillus*-like colonies were isolated from 207 soil samples of different sites (Lone et al. 2017). Captivatingly, soil environment could be inhabited by diverse microbial community and preferred as the main source for

Table 5 Median lethal concentration (LC₅₀) of *Bacillus thuringiensis* isolates against the third instar nymph of *Bemisia tabaci* (n = 20) on the 144 h of the concentration–response bioassay

Isolates	LC ₅₀ (spores/ ml)	95% CL		LC ₉₀ (spores/ ml)	95% CL		Slope ± SE	χ ²	P value
		Lower	Upper		Lower	Upper			
AAUDS-16	9.67 × 10 ⁶	1.30 × 10 ⁶	6.64 × 10 ⁷	3.9 × 10 ⁹	1.7 × 10 ⁹	5.6 × 10 ¹²	0.36 ± 0.07	3.102	< 0.01
AAUES-36A	3.20 × 10 ⁷	7.2 × 10 ⁶	2.29 × 10 ⁸	3.7 × 10 ¹⁰	2.2 × 10 ⁹	5.7 × 10 ¹³	0.42 ± 0.10	1.096	< 0.01
AAUES-69D	1.16 × 10 ⁷	2.83 × 10 ⁶	4.96 × 10 ⁷	6.0 × 10 ⁹	6.8 × 10 ⁸	8.5 × 10 ¹¹	0.47 ± 0.1	0.229	< 0.01
AAUMS-23	1.21 × 10 ⁸	1.45 × 10 ⁷	3.53 × 10 ⁹	4.7 × 10 ¹¹	2.2 × 10 ¹⁰	9.7 × 10 ¹⁸	0.28 ± 0.09	1.326	< 0.01

LC₅₀ average lethal concentration, CL confidence limit, SE standard error, χ² chi square

the isolation of *Bacillus* species in general and *Bt* in particular (Soares-da-Silva et al. 2015). The cultural characterization parameter preliminarily identified 40 isolates with typical colonial characteristics of *Bt*. In this result, the different isolates showed variations among one another in their colony color, margin, surface, and elevation. The different colony morphology of *Bt* was described as white to off-white colony color with irregular colony margin and flat to slightly raised colony elevation (Chai et al. 2016 and Padole et al. 2017).

Culturally characterized isolates were further subjected to morphological studies based on Gram staining and Coomassie brilliant blue staining as the suited parameters to distinguish *Bt* isolates from *B. cereus* group. All isolates were Gram-positive, spore-former, and rod-shaped bacteria. A similar result showed that *Bt* isolates were spore-former, rod-shaped, and gram-positive soil-inhabiting bacteria (Kassogué et al. 2015). The Coomassie brilliant blue staining analysis showed that from the total of 40 isolates, only 31 isolates were comprised of parasporal inclusion and classified as *Bt* whereas 9 isolates failed to have parasporal inclusion. Studies reported that crystal protein inclusions were the unique characteristic of *Bt* that distinguish *Bt* from other *Bacillus* species (Woldetenssay et al. 2009). In this crystal

morphology study, *Bt* isolates achieved 25.8% bipyramidal, 16.13% pyramidal, 25% spherical, 16.13% oval, 12.9% rod, and 6.5% amorphous crystal shapes. The crystal morphology variation was supported by Mukhija and Khanna (2018) who studied the crystal morphology of 140 *Bt* isolates and reported 52 (37.1%) bipyramidal, 31 (22.1%) pyramidal, 21 (15%) cuboidal, 18 (12.9%) round, and 18 (12.9%) amorphous crystal shapes. The differences in the crystal protein morphology distribution of isolates could be due to genetic variation caused by the difference in the environmental conditions.

The preliminary pathogenicity screening of entomopathogenic bacterial isolates was carried out by using *G. mellonella* larvae. From the total of 31 tested isolates, 20 (64.5%) isolates were virulent against *G. mellonella* with the percentage mortality of larvae ranged from 2.50 to 62.50%, 10 to 75%, and 20 to 95% within 48, 96, and 144 h post-application, respectively. There was a difference in toxicity against *G. mellonella* among tested isolates at $P < 0.01$. Bio-insecticidal evaluation of *Bt* revealed a significant reduction against *G. mellonella* larvae within 48 h post-infection (Grizanova et al. 2019). Research findings supported that *G. mellonella* larva was used as the best model host to study the interactions between insect pests and *Bt* (Dubovskiy et al. 2016). Moreover, the insecticidal

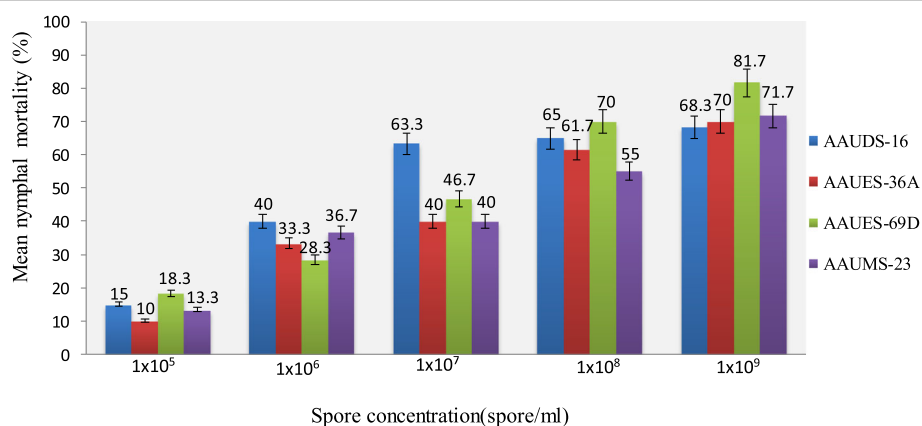


Fig. 4 Multiple concentrations caused percentage mortality response of spore-crystal *Bacillus thuringiensis* against *Bemisia tabaci* nymphs after 144 h of application

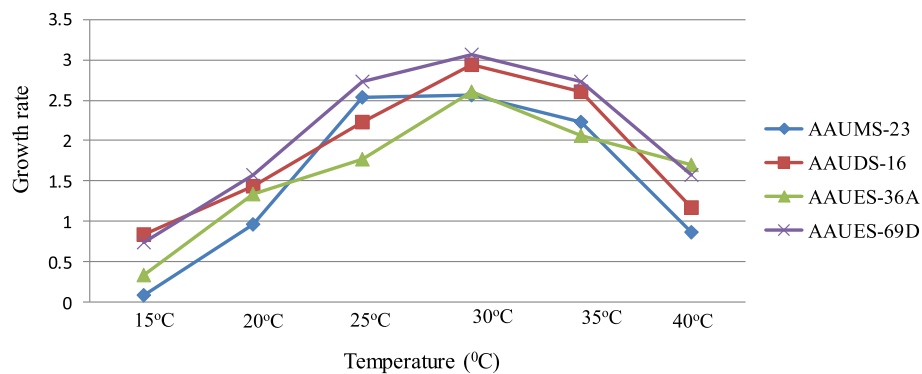


Fig. 5 The effects of various temperature on the growth rate (generations/h) of selected *Bacillus thuringiensis* isolates

potential evaluation of *Bt* isolates resulted in significant mortality against *G. mellonella* (Basedow et al. 2012) under laboratory and field conditions.

Molecular characterization of *Bt* is a vigorous technique than morphological, cultural, and physiological classification methods due to microbiological indistinguishable features of *Bt* isolates from other *B. cereus* groups (Abo-Bakr et al., 2020). As a result, 20 isolates screened from preliminary bioassay evaluation were selected for molecular characterization study. These entomopathogenic bacterial isolates were identified based on the *groEL* gene, and all isolates (100%) produced 533 bp amplified PCR products of *groEL* genes by using *B. cereus* group-specific primers of BalF and BalR. By the same token, the *B. cereus* group-specific primers (BalF and BalR) displayed 533 bp amplified products of *groEL* genes in detection and differentiation of *B. cereus* group isolates (Chang et al. 2003). According to Wei et al. (2018), *Bt* and other *B. cereus* group proficiently identified and evaluated with *groEL* gene sequences and its primers showed 56 (97%) amplified from 58 *B. cereus* isolates whereas, among 50 isolates, all (100%) isolates indicated amplified PCR products in *Bt*. As well, the *groEL* gene was a potential phylogenetic marker to adequately differentiate the species of *B. cereus* group compared to 16S ribosomal DNA gene sequence analysis (Chang et al. 2003).

The neighbor-joining examination showed that isolates of *Bt* from different soil sample sites of Ethiopia were closely related. The phylogenetic tree of indigenous isolates was constructed by neighbor-joining methods using the mega software. Phylogenetic analysis verified all bacterial isolates were positioned as part of the species of *Bt* as described by El-kersh et al. (2012).

Bioassay evaluation of 12 selected isolates of *Bt* against 3rd to 4th nymphal instars of *B. tabaci* with a concentration of spore-crystal suspensions (1×10^9 spores/ml) triggered 0 to 31.25%, 0 to 57.5%, and 0 to 82.5% percentage mortality within 48, 96, and 144 h, respectively.

The highest mortality was achieved by isolate AAUES-69D (31.25%) after 48 h, isolate AAUDS-16 (57.5%) after 96 h, and AAUES-69D (82.5%) after 144 h post-applications. This result in lined with the study stated that 34.84 to 51.21% mortality of *B. tabaci* nymphs after 48 h treated with different isolates of *Bt* (Ruiz-Sánchez et al. 2019). Salazar-Magallon et al. (2015) reported more than 92% 3rd to 4th nymphal instars of whitefly mortality after treated with culture broth containing spore-crystal complex. In Ethiopia, from the total of 130 isolates of *Bt*, 39 (30%) isolates revealed insecticidal properties against *Aedes africanus* (Ashenafi et al., 2001). The different isolates of *Bt* proved to successfully control insect pests by producing a wide range of insecticidal parasporal crystal proteins (Jurat-Fuentes and Crickmore, 2017).

Multiple concentration (1×10^5 – 1×10^9 spores/ml) response evaluations of most promising isolates of *Bt* were performed on *B. tabaci* nymphs under laboratory conditions for 144 h. The LC_{50} values of isolates were observed in the range of 9.67×10^6 to 3.32×10^7 spores/ml. Isolates of *Bt* AAUDS-16 reached the highest virulence, followed by isolate AAUES-69D with LC_{50} value of 9.67×10^6 and 1.16×10^7 spores/ml accordingly. The percentage mortality of *B. tabaci* was observed to increase with a subsequent increase in the concentrations of toxin-spore suspension of selected isolates of *Bt*. Correspondingly, Sivaji and Girija (2017) also described the increase in percent mortality of whitefly pests along with the increase in the toxin-spore concentration of *Bt* isolates. Significant differences were observed in the percentage mortality of the tested *B. tabaci* by different treatments. In the same way, variations in the effectiveness among different concentrations of *Bt* isolates were described (Patel et al. 2018).

The virulent isolates were tested for their growth response to various temperature ranges from 15 to 40 °C, and all of the isolates showed a maximum growth rate around 30 °C. Isolate AAUES-69D exhibited the

maximum growth rate of 2.07 generation/h, followed by AAUDS-16 (1.86 generation/h). Woldetenssay and Ashenafi (2009) studied the effects of temperature on the growth rate of isolates from different agroecological zones of Ethiopia, and the result indicated that isolates of *Bt* showed maximal growth rate at 30 °C even if all isolates grew well at a temperature ranged from 12 to 39 °C. Besides, the growth of *Bt* isolates was also proved at a different temperature range (25–50 °C), and the maximum growth rate of isolates showed between 30 and 35 °C (Abo-Bakr et al. 2020).

Generally, isolates of AAUES-69D and AAUDS-16 displayed high insecticidal potential against nymphs of *B. tabaci* with minimum LC₅₀ values of 1.16×10^7 and 9.67×10^6 spores/ml, and showed a maximum growth rate of 2.07 and 1.86 generation/h in a response to a wide range of temperature accordingly. These remarkable features of isolates are very important to screen, and they are shown to be competitive for future bio-pesticide applications for sustainable crop production under field conditions with a variable temperature range.

Conclusions

The present study showed the cultural, morphological, and bio-insecticidal diversity of *Bt* isolates detected from cultivated and forest soil samples. The insecticidal properties of *Bt* were evaluated against *G. mellonella* and *B. tabaci* nymphs. The percentage mortality effects of isolates showed a significant difference in both *G. mellonella* larvae and *B. tabaci* nymphs. Isolates of AAUDS-16 and AAUES-69D achieved the lowest LC₅₀ values and exhibited an average maximum growth rate at different temperature ranges. As a result, these isolates had the potential for biological control of whitefly and should be important candidates for different insecticidal toxin studies and use as gene sources for the construction of transgenic tomato plants.

Abbreviations

Bt: *Bacillus thuringiensis*; AAU: Addis Ababa University; CBB: Coomassie brilliant blue; PCR: Polymerase chain reaction; TAE: Tris-acetate-EDTA; NCBI: National Center for Biotechnological Information; EDTA: Ethylenediaminetetraacetic acid; UV: Ultraviolet; LC₅₀: Median lethal concentration; SE: Standard error; CL: Confidence limit; χ^2 : Chi square

Acknowledgements

The authors gratefully thank the Healthy Seedling Project granted by both the Ethiopian Biotechnology Institute and the Regional Project supported by the Austrian Development Agency (ADA) for financial support in this study.

Authors' contributions

AG planned the study, carried out the experiment, analyzed data, and wrote the manuscript. YC and FA supervised the overall activities in the study and editing manuscript. All authors read and approved the final manuscript.

Funding

Culture media, chemicals, reagents, substrates, stationary materials, and laboratory and field equipment were supplied by the Ethiopian Biotechnology Institute and the Regional Project.

Availability of data and materials

All data and material are stated in the manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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Received: 11 November 2020 Accepted: 28 January 2021

Published online: 09 February 2021

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