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Diversity and Characterization of *Bacillus thuringiensis* Strains and *Cry* Gene Variability in Agro-Ecological Zones of Mali

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aimed to identify the diversity of **:** *Bacillus thuringiensis* (*Bt*) strains in soils from different agro-ecological zones of Mali.
Study Design: Location and duration of the study: The work was carried out in Bamako, at LaboREM-Biotech located on the Badalabougou hill.
Methodology: Soil samples were collected in the different agro-ecological zones of Mali, specifically in the localities of Bougouni, Nièna, Sikasso, Koutiala, Ségou, Faya (classified forest) and Baguinéda. To properly carry out this activity, bacteria were isolated from soil samples. The different isolates obtained from the Luria Bertani (LB) agar culture medium were identified and characterized according to their macroscopic and microscopic aspects and their metabolic profile. After biochemical characterization, the different strains selected underwent molecular characterization by PCR using specific primers for the identification of Cry genes.
Results: In this study, seventy-two bacterial strains with a characteristic appearance of *Bt* were isolated. Sixty-nine (69) strains were identified as *Bt* after biochemical characterization of the selected isolates. Results of the PCR analysis showed that: among the *Bt* strains analyzed, sixty-two strains contained at least of the Cry1, Cry4, Cry10, Cry11, Cry24 and Cry32 genes and form 05 large groups with high diversity. Seven *Bt* strains selected did not code for any of the Cry genes sought.
Conclusion: Sixty-nine *Bt* strains were isolated in the different agroecological zones of Mali studied. These strains were used to strengthen the Bt bank already present at LaboREM-Biotech. This study shows the existence of a strong diversity of *Bts* in the agroecological zones studied and provides an overview of the diversity of cry genes carried by these strains and their importance in various fields.

Keywords: Bacillus thuringiensis; identification; crystals; genes.

1. INTRODUCTION

The protection of forests and crops against insect pests has led to the overuse of chemical pesticides. This use has harmful impacts on human, animal and environmental health and has proven to be ineffective against insects over time (Azize et al., 2021). Faced with this problem, biopesticides are the best alternative means of controlling insects. These biopesticides remain effective and less risky for the environment and human health (Šunjka and Mechora, 2022). As a result, Mali is a developing country with a majority of the population living in village communities, characterized by an organization where the different socio-economic units are located, with agricultural production as the main activity (Camara et al., 2023). Current agriculture must deal with many pests that are increasingly resistant to chemical phytosanitary product treatments. In addition, synthetic insecticides have shown harmful effects on human health and the environment. Explaining the need for their gradual withdrawal from the market to obtain protection that is both effective, environmentally friendly, reasonably priced and feasible from a socio-economic point of view. For this, the use of microorganisms against insect pests of crops is an alternative method to chemical control to be developed. It is in this context that bioinsecticides are prepared from living microorganisms.

Bioinsecticides are very specific, each one is only active against a limited number of species. They therefore respect the other species of the ecosystem and in particular the so-called auxiliary fauna, which takes part in the control of pest populations. Indeed, biological control applied by the use of bio pesticides "living organisms or products derived from these organisms having the particularity of eliminating or limiting crop pests", (Šunjka and Mechora, 2022). *Bacillus thuringiensis* (*Bt*) biopesticide is a mixture of spores and crystalline proteins that are toxic to certain insects. These proteins, called *Bt* toxins, can be used to control insect pests in agriculture. *Bacillus thuringiensis* biopesticides are considered environmentally friendly because they target specific pests and do not harm beneficial insects or other organisms. They are also less toxic to humans and animals than synthetic pesticides. *Bt* biopesticides can be applied in several ways, including spraying, dusting, and through genetically modified plants that express *Bt* toxins (Li et al., 2022). These *Bt* toxins are specific to certain groups of insects, such as Lepidoptera (butterflies and moths), Coleoptera (beetles), and mosquitoes that transmit diseases such as

dengue fever and malaria to humans (Kumari et al., 2022).

It is in this same context that the present study is part of the framework of assessing the diversity of *Bacillus thuringiensis* strains in certain agroecological zones of Mali. This activity therefore aims to (i) isolate *Bacillus thuringiensis* from different agroecological soils, (ii) determine the microbiological and biochemical characteristics of the isolated *Bacillus thuringiensis* isolates, and (iii) identify the Cry genes of *Bacillus thuringiensis* isolates.

2. MATERIALS AND METHODS

2.1 Study Sites

Soil samples were collected in the different agroecological zones of Mali, specifically in the localities of Bougouni, Nièna, Sikasso, Koutiala, Ségou, Faya (classified forest) and Baguinéda. Three composite samples were formed in each locality for each ecological zone (never cultivated fields, fallow fields and cultivated fields) of each.

2.2 Sample Collection and Preparation

Samples composed of 500g of soil were collected from the different sites with a small hoeing pick and sampler. At the sampling site, solid waste (wood twigs, dead leaves, etc.) was swept before taking samples and placed in plastic bags previously sterilized and transported to the laboratory where they were stored in a refrigerator at 4 ° C before analysis.

2.3 Sample Analysis

2.3.1 Isolation of *Bacillus thuringiensis*

To isolate *Bacillus thuringiensis* from the soil samples collected, a combination of the selective salt solution method described by Valicente et al. (2008) and the heat treatment method was used.

2.3.2 Heat treatment of samples

Thus, 1 gram of each sample was weighed using a PioneerTM precision balance and aseptically placed in test tubes containing 5 mL of sterile saline solution (8 g of Nacl / l). The different samples thus treated were stirred at 250 rpm for 16h. Then, 1 mL of supernatant was taken from each sample and transferred to sterile test tubes. The tubes containing the supernatant underwent a thermal shock at 65 ° C for 30 minutes in a water bath. This treatment allows excluding most of the non-sporulated vegetative cells of bacteria (Travers et al. 1987).

2.3.3 Inoculation of the culture medium by the samples

The solutions obtained after the thermal shock were considered as stock solutions. The stock solution of each sample underwent a decimal dilution up to 10-4. Two drops of each stock solution and of each of the dilutions were spread on Luria Bertani (LB) culture medium. One liter of LB medium is composed of: 1.0 g of glucose, 8.0 g of nutrient broth, 0.02 g of FeSO4, 0.02 g of ZnSO4, 2.0 g of yeast extract, 0.03 g of MnSO4, 0.3 g of MgSO4, 10.0 g of tryptone, 5.0 g of NaCl, 12.0 g of agar and adjusted the pH to 7.5. This experiment was repeated twice and uninoculated control plates were reserved for each sample. The culture media thus inoculated were inverted and placed in an incubator (Incucell) at 30 ° C for 24 and 48 hours, which allowed the germination of *Bt* spores. (Travers et al. 1987; Kassogué et al. 2015).

2.3.4 Counting *Bacillus thuringiensis* (*Bt*) colonies on Petri dishes

After 24 hours of incubation, the number of colonies on each dish was counted using the FUNKE GERBER colony counter. Dishes with a number of colonies between 30 and 300 were used to calculate the number of colony-forming units per gram (CFU/g) while taking into account the amount of soil used to prepare the solution, the dilution used and the volume of sample spread (Kassogué et al. 2015).

2.3.5 Purification of *Bacillus thuringiensis* (*Bt*) colonies

The different bacterial colonies that were grown on L.B agar medium and showing a morphological appearance characteristic of *Bt* were purified by the solid medium exhaustion method. That is to say, each colony was streaked on solid LB medium until pure colonies were obtained. To confirm colony purification, each pure colony was successively sub-cultured onto LB medium containing ampicillin at a final concentration of 10 µg/ml (Yoo et al. 1996). The treated plates were then inverted and incubated in an incubator at 30°C for 24 hours.

2.4 Characterization of Isolated Strains

2.4.1 Macroscopic and microscopic characterization of microbial cultures

2.4.1.1 Macroscopic characteristics

The macroscopic observation was done with the naked eye. During this analysis, the appearance, consistency, shape, color, and size of the colonies were determined (Kassogué et al. 2015).

2.4.1.2 Microscopic characteristics

Observation in the fresh state: The fresh state examination was done to see if the microorganisms are mobile or not. This technique was also very useful to observe the grouping mode of the microorganisms. To carry it out, a small amount of the bacterial culture was taken using a sterile transfer pipette, placed on a slide then covered with a coverslip and observed directly under the Motic® optical microscope (Kassogué et al. 2015).

Observation after Gram staining under optical microscope: For this microscopic analysis of the isolates, the staining technique described by Eswarapriya et al. (2010) was tested.

Observation after Coomassie blue staining under microscope: To highlight the presence of crystals, the Coomassie blue staining technique represented by Sharif et al. (1988) was used. Therefore, the smears prepared from the bacterial isolates were stained with a Coomassie blue solution (0.25% Coomassie blue, 50% ethanol, 7% acetic acid) for 3 minutes, washed with water, dried and visualized under optical microscope. With this method, the released crystals are stained purple and are easily differentiated from the spores. The spores are colorless and oval or round in shape depending on the species in question. Vegetative cells appeared as purple colored rods.

Observation after malachite green staining under the microscope: To determine the presence of endospores in the different isolates, a small portion of bacterial isolate was spread on the slide using an inoculation loop and diluted with a drop of sterile water and flame dried. After drying, the slides were flooded with malachite green (5g of malachite green dissolved in distilled water and the volume was adjusted to 100ml) and immediately heated until vapors were emitted. The slides were cooled and then reheated again (the operation should last 10 minutes in total). After cooling, the slides were rinsed with water and counterstained with safranin O (0.5g of safranin O powder dissolved in distilled water and the final volume was adjusted to 100ml) for 2 minutes, then rinsed with water. The slides were air-dried and observed under an optical microscope with a 100X objective with immersion oil (Kassogué et al. 2015; Verma et al. 2018).

2.4.2 Biochemical characteristics

2.4.2.1 Catalase test

The catalase test made it possible to highlight bacteria that possess this enzyme, catalase (Kassogué et al. 2016). This test made it possible to distinguish bacteria with the same cell morphology and the same Gram staining. To perform this test, the different isolates were deposited directly on a slide. Then, using the Pasteur pipette, a drop of hydrogen peroxide (H_2O_2) was placed on the bacterial growth. A release of gas (the formation of bubbles) corresponds to the presence of catalase. The absence of bubbles indicated the non-production of catalase.

2.4.2.2 Oxidase test

The search for cytochrome oxidase was carried out using "Ox" disks whose reaction zone is composed of filter paper impregnated with N, N-dimethyl-1,4-phenylenediamine-dichloride. From a solid medium, part of the colony was deposited on an oxidase disk placed on a slide, using a buttoned Pasteur pipette. The presence of cytochrome oxidase results, in 20 to 60 seconds, in the appearance of a red color rapidly turning very dark purple (Kassogué et al. 2016; Joffin et al. 2006).

2.4.2.3 Esculin hydrolysis:

This test was performed on the agar medium containing esculin bile. After incubation of the culture at 30°C for 24 hours, the hydrolysis of esculin releases the aglycone which is detected by a chemical reaction in the presence of iron salt and gives a black color to the culture medium (Kassogué et al. 2015; De VOS et al. 2009).

2.4.3 Identification of Cry genes of *Bacillus thuringiensis* isolates by PCR

2.4.3.1 Extraction of bacterial DNA

The genomic DNA of the bacteria was extracted using the Promega WIZARDR Genomic DNA Purification kit. The concentrations of the DNA extracts were determined using an Eppendof spectrophotometer. Samples were diluted to 20ng/µl, with Water Nuclease Free VWR and stored at -20°C.

2.4.3.2 DNA amplification of different strains of Bacillus thuringiensis: PCR

DNA samples were amplified with 6 pairs of SSR marker primers chosen on the basis of the presence of crystal proteins called delta-endotoxins or Cry pro-toxins, which have an effective biopesticidal property. This PCR was done according to number are reported in Table 1 (Vianaa et al. 2020).

2.4.3.3 Preparation of the reaction mixture

The reaction mixture with a total volume of 15µL was prepared with the Promega PCR kit reagents, the Cry primers and the DNA extracts under the ASALAIR Laminar Flow 900-FLY hood. It was distributed in the PCR tubes (trips of 8 PCR Tubes). A negative control (reaction mixture without DNA) was created for each PCR reaction. The presence of a band in the control indicates contamination. The composition and proportions of the reaction mixture are shown in Table 2 (Vianaa et al. 2020).

Table 1. Cry name, sequences of Cry primers used and hybridization temperature

N°	Name	Séquences 5'---3'	Hybridization température
1	*Cry1 F*	CTGGATTTACAGGTGGGGATAT	52°
	Cry1 R	TGAGTCGCTTCGCATATTTGACT	
2	Cry4 F	GCATATGATGTAGCGAAACAAGCC	50°
	Cry4 R	GCGTGACATACCCATTTCCAGGTC	
3	*Cry10 F*	TCAATGCTCCATCCAATG	51°
	Cry10 R	CTTGTATAGGCCTTCCTCCG	
4	Cry11 F	TTAGAAGATACGCCAGATCAAGC	51°
	Cry11 R	CATTTGTACTTGAAGTTGTAATCCC	
5	Cry24 F	TTATCAATGTTAAGGGATGC	52°
	Cry24 R	ACTGGATCTGTGTATATTTTCCTAG	
6	Cry32 F	TGGTCGGGAGAGAATGGATGGA	48°
	Cry32 R	ATGTTTGCGACACCATTTTC	

Table 2. Composition of the reaction mixture

	Vi	Ci	Vf	Cf
H_2O	7,15µl			
Go Taq Green Master Mix	12,5µl	5X		2,5X
Amorce allée F	1µl	100pmol/µl	25µl	4pmol/µl
Amorce retour R	1µl	100pmol/µl		4pmol/µl
ADN	3,33µl	20ng/µl		2,66ng/µl

VI : Initial volume: Vf : Final volume, Ci: Initial concentration, Cf: Final concentration.

Table 3. Program of the DNA amplification

Steps	Temperature (°C)	Time (Minute)
Initial denaturation	94	5
Denaturation	94	1
Hybridization	48- 52	1
Elongation	72	1
Final elongation	72	7
Conservation	04	∞

Denaturation, hybridization and elongation constitute a cycle that repeats 35 times

2.4.3.4 DNA amplification program

The PCR tubes containing the reaction mixture were vortexed using the VWR INTERNATIONAL Vortex to homogenize the contents. They were then centrifuged with the benchmark microcentrifuge and introduced into the 2720 applied System Thermocycler. The program below was applied (Table 3).

2.4.3.5 Electrophoresis and visualization of PCR products

Ten microliters (10µl) of PCR products were migrated on a 2% (weight/volume) Ultra-Pure agarose gel (recommended for the separation of DNA fragments smaller than 100bp) at 100V for 2h 00 min. The gel was prepared with a solution of TBE Buffer AppliChen 0.5X (Tris, Borate acid, EDTA), 30µl of ethidium bromide 10% (1mg/ml) visualized and photographed under UV with E-BOX VX2 gel analysis darkroom version 15.06.

Data analysis: The size of the bands on the agarose gel was determined in base pairs with the E-Capt software version 15.06 by comparison with the standard Promega 100bp DNA Step Ladder marker (Vianaa et al. 2020). The genetic distance was calculated using the Power Marker 3.25 software. The phylogenetic tree based on the genetic distance was constructed with the same software, displayed and edited with the MEGA software version 7 (Dao et al., 2018).

3. RESULTS

3.1 Collected Samples

A total of 18 samples were collected from several locations in three different agroecological zones. The locations, agroecological zones and GPS coordinates of the soil samples collected from the different agroecological zones are recorded in Table 4.

3.2 Microbiological Analysis of Collected Samples

3.2.1 Distribution of isolated bacterial strains

The results of analysis of soil samples from different agroecological zones are reported in Table 5.

3.2.2 Morphological characters of the isolated strains

3.2.2.1 Macroscopic and microscopic characters of microbial cultures

Macroscopic character: After purification, isolates with the characteristic aspects of *Bacillus thuringiensis*, flat, slightly raised, white or whitish colonies, with regular borders, smooth surfaces, a creamy consistency were retained. Seventy-two isolates had a *Bt* appearance. The different colonies were purified and named using the first and second letters of the genus and species respectively: B for Bacillus and t for *thuringiensis*. In addition, two numbers, representing respectively the sample number and the sequence number of the isolates.

The photographs in Fig. 1 give an overview of colonies of some of our isolates.

Microscopic characteristics: Observation in the fresh state and Observation after Gram staining under an optical microscope.

The fresh state examination was carried out to check the mobility, shape and size of the microorganisms. In the fresh state, all the isolates showed a Bacillus shape with the exception of BP3.1; BP3.2 and SéP15.2 which were in the form of a shell and which were eliminated from the list of isolates retained. The photographs in Fig. 2 give a bacterial overview of some isolates after Gram staining. A collection of 69 isolates was retained for further characterization. Then, all the isolates were Gram positive (Fig. 2).

Aspects after Coomassie blue staining under light microscope: The Coomassie blue staining technique showed blue colored crystals while the spores were oval, central and colorless (Fig. 3).

Aspects after staining with malachite green using an optical microscope: Indeed, the observation showed that the spores are oval and colored green while the vegetative cells are colored pink (Fig. 4). These characteristics of the spores correspond to those of *B. thuringiensis*. All the strains selected had spores; meeting the characteristics of *B. thuringiensis.*

3.3 Biochemical Characteristics of the Selected Strains

The results of the KOH test, production of catalase, oxidase and escullinase are recorded in Table 6.

3.4 Molecular Characteristics of the Isolated *Bacillus thuringiensis* Strains

The profiles of the different fragments obtained after PCR amplification showed that eleven (11) *Bt* had the crystal protein gene (Cry1), fifty (50)

Bt for Cry4, seventeen (17) *Bt* for Cry10, twenty-one (21) for Cry11, thirty-three (33) *Bt* for Cry24, twenty-nine (29) *Bt* for Cry 32. The results also showed that 11 strains have at least four Cry genes simultaneously. Among the sixty-nine (69) strains analyzed, seven (7) did not code for any Cry (NC5.1; NP6.5; SC9.2; SéJC13.2; SéP15.6; KlJC16.2; BaP18).

Table 4. Soil sampling site

N° Ech	Sampling sites	Ecological zones	GPS coordinates	Sampling date
1	Bougouni	Field never cultivated	Lat: 11,409392 Long: -7,53211	31/03/2021
2	Bougouni	Cultivated field	Lat: 11,389148 Long: -7,528657	31/03/2021
3	Bougouni	Fallow	Lat: 11,434175 Long: -7,501452	31/03/2021
4	Niena	Field never cultivated	Lat: 11,388785 Long: -6,280637	31/03/2021
5	Niena (bafon)	Cultivated field	Lat: 11,407035 Long: -6,325618	31/03/2021
6	Niena	Fallow	Lat: 11,416062 Long: -6,333432	31/03/2021
7	Sikasso	Field never cultivated	Lat: 11,409392 Long: -5,743985	01/04/2021
8	Sikasso	Cultivated field	Lat: 11,314445 Long: -5,661700	01/04/2021
9	Sikasso	Fallow	Lat: 11,314445 Long: -5,661700	01/04/2021
10	Koutiala (kouoro barrage)	Field never cultivated	Lat: 12,026073 Long: -5,690920	01/04/2021
11	Koutiala (Nièsoumana)	Cultivated field	Lat: 12,583973 Long: -5,613722	01/04/2021
12	Koutiala	Fallow	Lat: 12,335467 Long: -5,469755	01/04/2021
13	Ségou (Bani)	Field never cultivated	Lat: 13,214000 Long: -5,904812	02/04/2021
14	Ségou	Cultivated field	Lat: 13,186703 Long: -6,499687	02/04/2021
15	Ségou (Fleuve Niger)	Fallow	Lat: 13,408663 Long: -6,330802	02/04/2021
16	Koulikoro (Faya)	Field never cultivated	Lat: 12,672312 Long: -7,475467	02/04/2021
17	Baguineda	Cultivated field	Lat: 12,624217 Long: -7,778040	02/04/2021
18	Baguineda	Fallow	Lat: 12,576642 Long: -7,796537	02/04/2021

Ech: Sample; Lat: Latitude; Long: Longitude

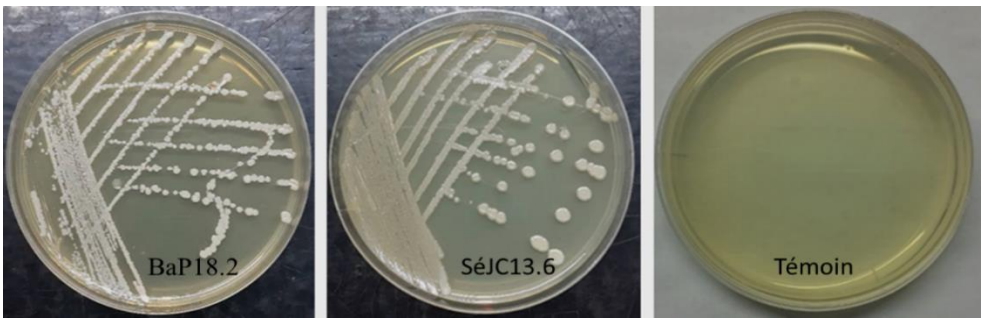

Fig. 1. Observation of colonies of isolates BaP18.2; SéJC13.6 and the control

Table 5. Distribution of *Bacillus thuringiensis* isolated from different soil samples collected in different agroecological zones

Sites	Sampling zones	Samples	Number of bacteria	Number of *Bt*	*Bt* index (%)
Bougouni	Field never cultivated	1	460	2	0,43
	Cultivated field	2	1775	7	0,39
	Fallow	3	2160	5	0,23
Niena	Field never cultivated	4	610	2	0,32
	Cultivated field	5	2280	5	0,21
	Fallow	6	1200	6	0,50
Sikasso	Field never cultivated	7	2670	5	0,18
	Cultivated field	8	1560	1	0,06
	Fallow	9	720	2	0,27
Koutiala	Field never cultivated	10	660	2	0,30
	Cultivated field	11	1080	3	0,27
	Fallow	12	100	2	2,00
Ségou	Field never cultivated	13	1960	12	0,61
	Cultivated field	14	1480	3	0,20
	Fallow	15	1000	7	0,70
Koulikoro	Field never cultivated	16	370	3	0,81
Baguineda	Cultivated field	17	1360	2	0,14
	Fallow	18	1240	3	0,24

The analysis results showed that Bt is found in all the agroecological zones studied; with an index between 0.06 and 2. In total, seventy-two Bt isolates were retained during this activity.

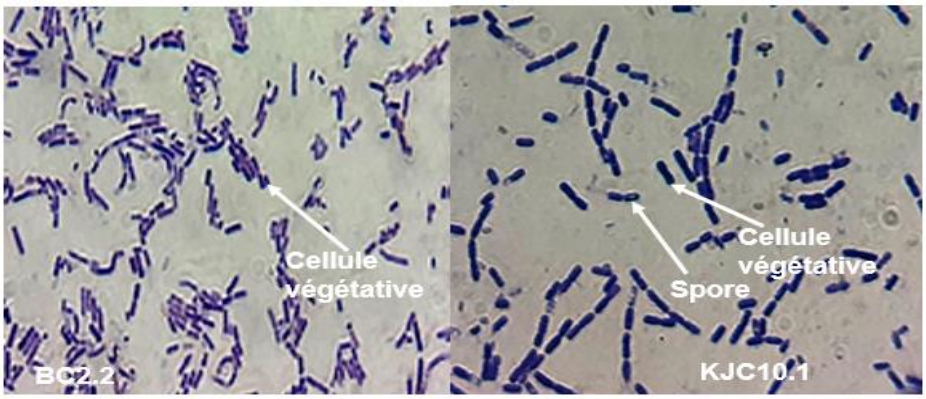

Fig. 2. Photograph of isolates BC2.2 and KJC10.1 after Gram staining under G×1000 light microscope

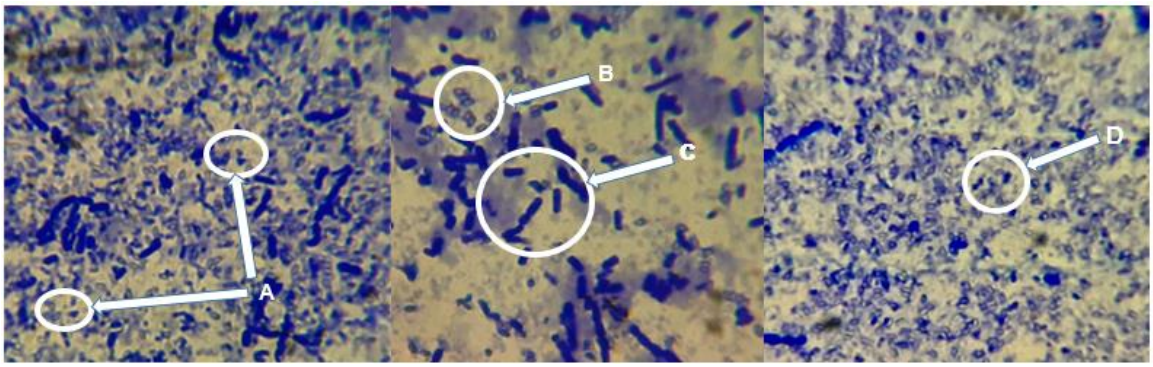

Fig. 3. Photograph of some isolates after staining with Coomassie blue using a phase contrast microscope (A: Bipyramidal crystals; B: Spores; C: Vegetative cells; D: Spherical crystals)

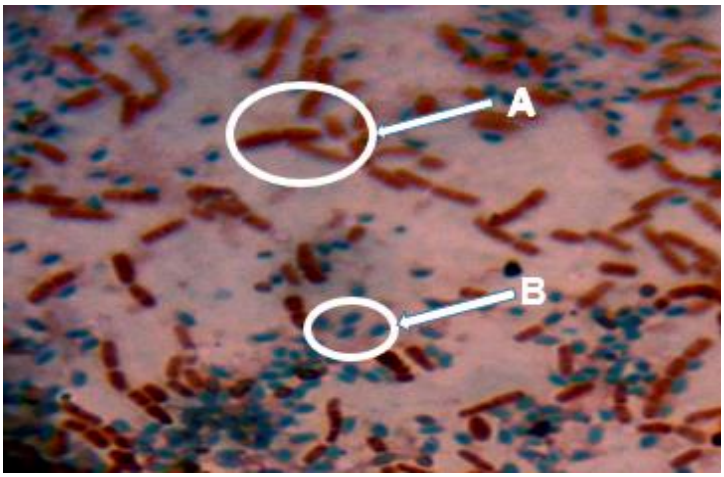

Fig. 4. Observation under optical microscope (Gx100), of spores (B) and vegetative cells (A) of isolate KJC10.2 after staining

Table 6. Results of KOH test, production of catalase, oxidase and escullinase by the selected strains of *Bacillus thuringiensis*

Test	*Bt* strains	Percentage of positivity (%)
KOH	All negative	100
Catalase	All positive	100
Oxidase	All positive except BC2.1; BC2.2; BC2.4; BC2.5 et BaP18	92
Escullinase	All positive except BJC1.2; BC2.3; BP3.4; NC5.4; SJC7.5; SJC7.6; SP8.1; SC9.1; SC9.2; KJC10.2; KP12.2; SéJC13.2; SéJC13.5; SéJC13.7 et SéJC13.8	78

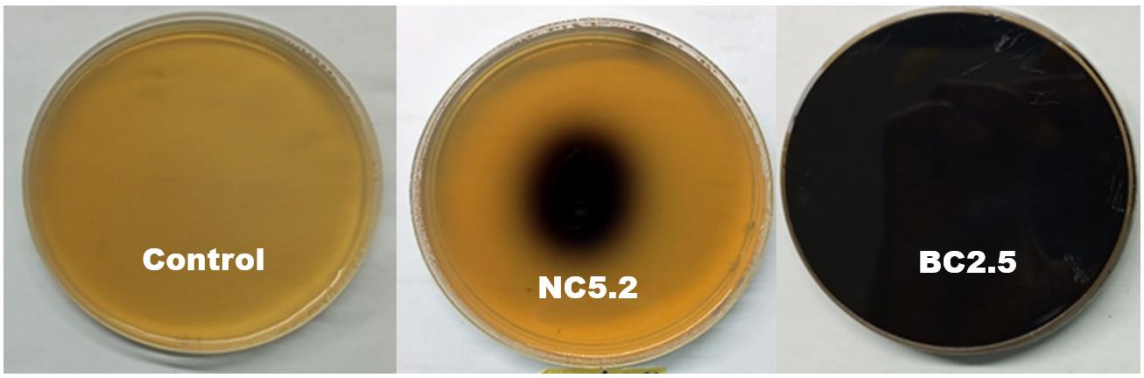

Fig. 5. Hydrolysis of esculin by some strains after 24 h of incubation

Table 7. Percentage of *Bt* strains with different Cry genes across field types

Field Types	Percentage of Cry genes					
	Cry1	Cry4	Cry10	Cry11	Cry24	Cry32
Field never cultivated	15	73	19	27	58	42
Cultivated field	20	84	32	36	56	52
Fallow	9	61	19	31	33	38

Cry4 and Cry24 were the most represented in the never cultivated fields with respective frequencies of 73% and 58%. This predominance of the same genes was observed in the cultivated fields, 84% and 56% respectively. While in the fallow (near) fields, Cry4 and Cry32 were more frequent

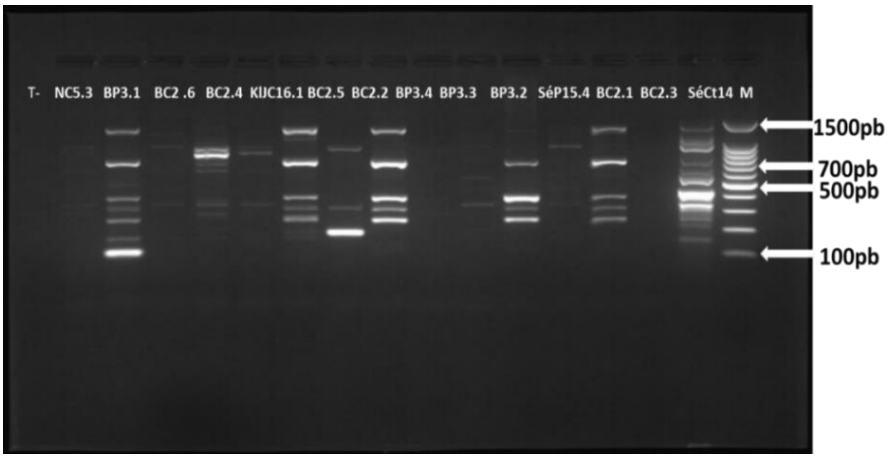

Fig. 6. Migration profiles of some *Bt* strains with the Cry4 marker. NC5.3, BP3.1, BC2.6, BC2.4, KIJC16.1, BC2.5, BC2.2, BP3.4, BP3.3, BP3.2, SéP15.4, BC2.1, BC2.3, SéCt14: *Bacillus thuringiensis*

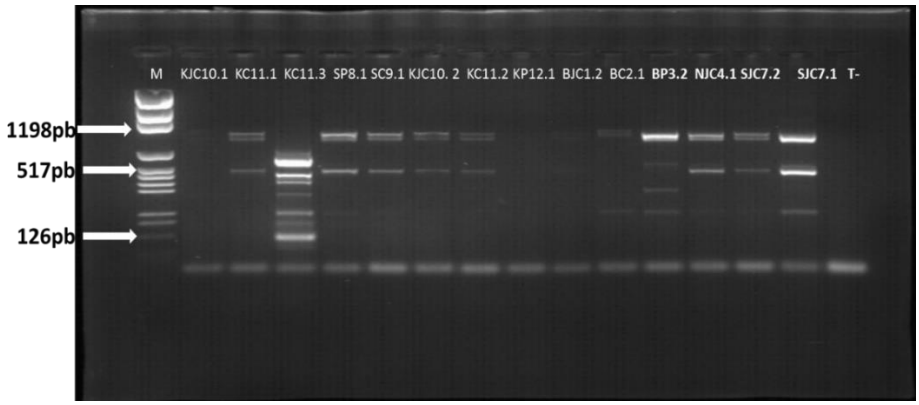

Fig. 7. Migration profiles of some *Bt* strains with the Cry32 marker. KJC10.1 KC11.1 KC11.3 SP8.1 SC9.1 KJC10. 2 KC11.2 KP12.1 BJC1.2 BC2.1 BP3.2 NJC4.1 SJC7.2 SJC7.1

Table 8. Percentage of *Bt* strains possessing the different Cry genes according to the regions

Regions	Cry genes					
	cry1	Cry4	Cry10	Cry11	Cry24	Cry32
Bougouni	33	83	66	58	66	58
Sikasso	9	52	41	28	38	47
Koutiala	0	85	0	42	85	71
Ségou	19	85	9	19	42	28
Koulikoro	12	62	25	12	25	12

3.4.1 Frequency of *Bt* strains with different Cry genes across field types

The diversity of *Bt* strains with different Cry genes across field types is shown in Table 7.

3.4.2 The frequency of *Bt* strains possessing the different Cry genes according to the regions studied

The diversity of *Bt* strains possessing the different Cry genes in different agro-ecological zones is recorded in Table 8.

After analysis, Table 8 showed All forms of Cry genes studied were present in the Bougouni region and the frequency was greater than 50% with the exception of Cry 1 which had a frequency of 33%. Similarly, in the Sikasso, Segou and Koulikoro regions, the presence of all forms of Cry genes was observed but frequencies lower than 50%, except for the Cry 4 gene. In the Koutiala region, Cry 1 and Cry10 were absent.

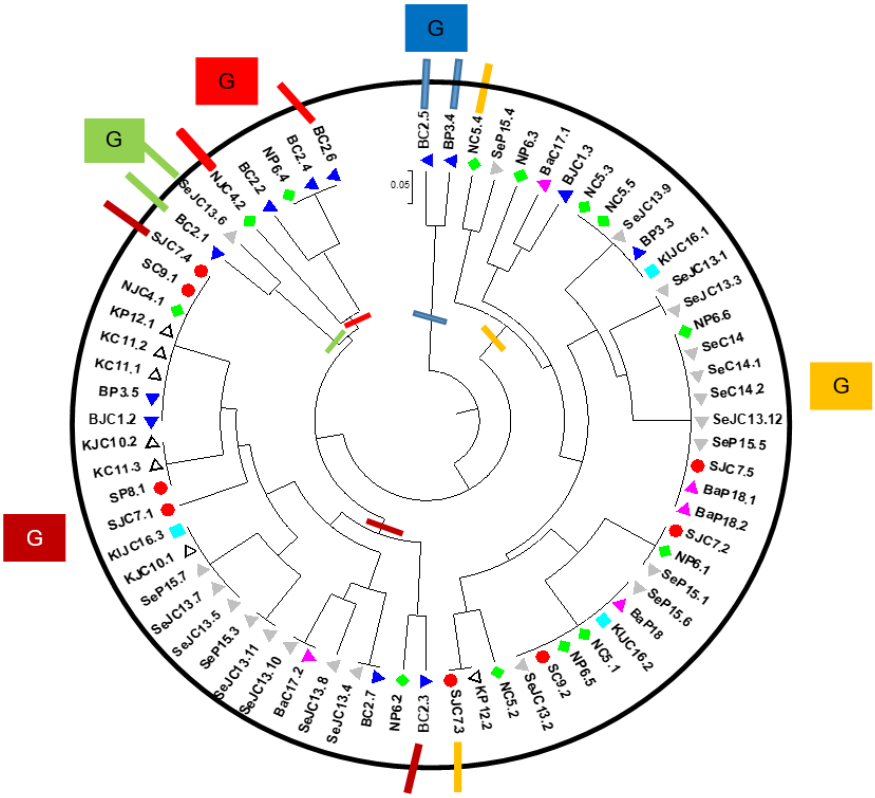

Fig. 8. Phylogenetic tree of strain

3.4.3 Phylogenetic relationships of bacteria possessing Cry genes

The previous results and using the MEGA-SAT software made it possible to establish the phylogenetic tree. The analysis of the similarity of *Bt* strains according to the Cry genes made it possible to classify the strains into five groups. Group 1 (G1) was composed only of *Bt* strains from the Bougouni region. Group 2 (G2) was formed by 54% of the strains collected in the different regions and was composed of five subgroups. Group 3 (G3) was composed of 41% of the *Bts* of the collection studied. On the other hand, group 4 (G4) was formed with two *Bts* strains from the Bougouni and Ségou region. Group 5 (G5) contained five *Bt* strains from Bougouni and Niéna. The composition of the different groups obtained is represented by the dendrogram (Fig. 8).

4. DISCUSSION

The bacterial distribution results of this activity showed a *Bt* index ranging from 0.06 to 2. The Bt indices found are different between the sampling sites of each sample. This means that all soils in Mali contain *Bt* but at different concentrations. Shirshir et al. (2014) and Ammouneh et al. (2011) obtained a *Bt* index of 0.125 from soil samples collected in different regions of Syria. This value is included in the different indices obtained in our study. Kassogué et al. (2015) obtained indices between 0.005 and 0.117, during the characterization of *Bacillus thuringiensis* isolates with insecticidal activities from areas where maize is mainly grown. Shishir et al obtained in Syria a *Bt* index of 0,12 which is in the range obtainTheir results are significantly lower than ours. In a study of characterization and evaluation of the pathogenicity of *Bacillus thuringiensis* in the western province of Azerbaijan in Iran, Aramideh et al. (2010) obtained *Bt* indices ranging from 0.041 to 0.147, which is significantly lower than our results. This difference in index can be explained by the fact that the samples were taken from several different agroecological and climatic zones of Mali.

Our work after staining with coomassie blue revealed that all the strains retained had crystals, but with different shapes. Some strains produced only one shape of crystals; either spherical, cubic, bipyramidal or pyramidal, but others produced a combination of several crystal

shapes at the same time. This result confirms that of Fané et al. (2017) obtained during their research work, thirty-five (35) strains of *Bacillus thuringiensis* that produced protein crystals (δ-endotoxins). Three types of crystals (pyramidal, spherical and cubic) were found alone or in association. In addition, we obtained by PCR six genes encoding 6 12 different crystal (Cry) endotoxins from the isolated *Bacillus thuringiensis* strains. Arsowv et al. (2023) obtained 12 cry genes encoding 12 different crystal endotoxins (Cry) by PCR with specific primer pairs, twice as much as we got. Out of the the studied *Bacillus thuringiensis*; eleven (11) had the gene encoding the crystal protein (Cry1), fifty (50) the gene cry4, seventeen (17) the cry10, twenty-one (21) the cry11, thirty-three (33) the cry24, and twenty-nine (29) the cry 32. The results also showed that 11 isolates had at least four Cry genes simultaneously. Among the sixty-nine (69) isolates analyzed, seven (7) *Bt* isolates did not code for any Cry (NC5.1; NP6.5; SC9.2; SéJC13.2; SéP15.6; KlJC16.2; BaP18). This diversity of Cry genes was confirmed by Fané et al. (2017) during her studies to determine cry1B, cry1C, cry1F and cry2 genes on local isolates from soil samples from areas where corn is grown par excellence. They obtained after the gene amplification technique by PCR the desired fragments and several other unexpected fragments on the agarose gel. In addition to the presence and diversity of cry genes, Raha et al. (2017) showed the presence, in their isolates, of *Bt* without no cry gene. This work also showed that the isolates identified in the different areas presented very diverse profiles and would indicate new genes within the *Bacillus thuringiensis* isolates, as noted by Fané et al. 2017. This revelation of Cry genes was confirmed by Ammouneh et al. (2010) during their first study in Syria on the identification of local isolates of *B. thuringiensis* from soils in different regions.

The phylogenetic tree obtained during this activity made it possible to classify the *Bt* isolates retained from different agroecological zones into 05 groups based on the Cry genes. Kebdani (2017), during his thesis on the identification of local isolates of *Bacillus thuringiensis* for biological control against *Ceratitis capitata* and other pathogens of the orange tree (*Citrus sinensis*) also developed the phylogenetic tree whose predominant species was *Bacillus thuringiensis* which represented 50% of the isolates identified during this work.

5. CONCLUSION

This work shows that *Bacillus thuringiensis* represent only a very small proportion of the total microflora of the soils studied and their index varies from one agro-ecological zone to another. The agro-ecological zones of the five (05) regions showed a diversity of *Bt* linked to the presence of Cry genes. They could be considered as potential reservoirs of species to be used as biopesticides. The macroscopic and microscopic characterization made it possible to select 69 isolates of *Bacillus thuringiensis*. After the molecular characterization, the sixty-nine (69) *Bt* isolates obtained showed that they possessed the crystal protein gene (Cry); except seven (7) *Bt* isolates did not code for any Cry.

The identification of the Cry genes studied made it possible to develop the phylogenetic tree, composed of 05 large groups. All the *Bt* strains selected were taken to constitute a bank

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

The authors hereby declare that no generative AI technologies such as large language models (ChatGPT, COPILOT, etc.) and text-to-image generators were used in the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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