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Genetic Diversity of Native *Bacillus thuringiensis* Strains Isolated from Soil of Different Localities in Mali and their *cry* Gene Profile

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RF, AHB, FHV and DT designed the study, wrote the protocol and performed the statistical analysis. Authors RF, FS and AK wrote the first draft of the manuscript. Authors RF and DT managed the analyses of the study. Authors AH and AHD managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine the genetic diversity of *Bacillus thuringiensis* strains isolated from soils of different locality in Mali, and select strains with *cry1F, cry1B* and *cry1C* genes to control caterpillars and strains with *cry2* gene against African rice gall midge.
Study Design: Strains of *Bacillus thuringiensis* (Bt) used in this study belong to collection of Laboratory of Research in Microbiology and Microbial Biotechnology (Laborem-Biotech) and that

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isolated from different ecological environment of Mali. Bt strains used as positive controls were kindly provided by Dr. Fernando Hercos Valicente from Embrapa Milho e Sorgo (Brazil).
Methodology: The total DNA of the native *B. thuringiensis* and reference strains were extracted from overnight grown culture. Gene identification was performed by amplification (PCR) of DNA of Bt strains using specific primers. The gel revelation was performed using ethidium bromide and the gel photography was performed using an E-Box VX2 system, Version 15.06.
Results: *Native B. thuringiensis* strains studied, showed high genetic diversity. 48,3% of the studied *B. thuringiensis* strains of the collection carry cry1 gene, 49,06% of them harbor both *cry1* and cry2 gene, 5.7% of the native Bt strains didn't react with any *cry1* and *cry 2* specific primers and 94.3% of the strains produce different PCR products. The analysis of cry1 positive *B. thuringiensis* showed sub-group frequencies of 7.6% for *cry1*F and 3.8% for *cry1*B and *cry1*C. In native Bt strains of our collection, the *cry2* gene was always present with one or two cry1 sub-group(s).
Conclusion: In this study, high genetic diversity in the native Bt strains from the bacterial collection in Mali was observed. Most of the native Bt strains studied harbor the *cry1* gene only. In the *cry1* gene profile, the *cry1F* sub-group was found to be the most frequently detected. None of studied Bt strains harbored the *cry2* gene only.

Keywords: Bacillus thuringiensis; cry gene; cry proteins; diversity; Helicoverpa armegira; mali.

1. INTRODUCTION

Mali is an agricultural country where the use of chemical insecticides against insect pests has significantly increased the productivity of both agricultural and forestry services [1].

It has been proven that maize and rice in Mali are heavily attacked by pests; particularly *Helicoverpa armegira* and *Orselia oryzivora* [2]. Increase production and the marketing of rice and maize, is crucial in meeting small farm-families' food demands and reducing poverty. With an aim of controlling these insect pests and improving rice and maize production, chemical treatments were the method most used in West Africa. The high prices and the harmful effects of synthetic pesticides on the environment and consumer's health limit their use on food crops by poor small farmers. Actually, agriculture needs to be very productive and less polluting. Thus, the international agreements, like the protocols of Montreal and Rio de Janeiro, converge to limit the use of chemical pesticides. Growing concerns of the public about the potentially harmful effects of the use of chemical insecticides for the environment, led the scientific community to look for alternatives to chemical control [1]. But, to date, adequate products and technology to biologically control these insect pests are crucially lacking in West African countries, mainly in Mali, hence an important *Bacillus thuringiensis* (Bt) collection exist.

For some years *Bacillus thuringiensis* (Bt) is used to control different species of crop pests, and and it has been proven that *B. thuringiensis* with cry1F, cry1B and cry1C and those with cry2 gene produce toxins highly efficient active against Lepidoptera. The efficiency of insecticidal properties of *B. thuringiensis* is due to the synthesis of the crystal proteins encoded by the *cry* genes [3]. Currently, more than 560 *cry* genes have been identified and classified into 68 classes based on the homology of their proteins [4]. *Cry* proteins presenting toxic activities against the larvae of several insects (Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, and Malophaga orders) have been described [5-11]. *B. thuringiensis* have been found also active against other organisms, including platyhelminths, nematodes, protozoa [12,13].

The polymerase chain reaction (PCR) is used to identify specific cry genes and characterize Bt strains [14,6,15]. It may also be used to predict insecticidal activity [16], determine ecological distribution [14,17], and identify new cry genes [18,19]. Besides being an important component of studying Bt resources, the characterization of Cry proteins and its genotypic composition may help understanding its insecticidal activity.

The present investigation aimed to: (i) study the genetic diversity and cry (cry1 and cry2) gene profile of native *Bacillus thuringiensis* strains isolated from different soils of Mali and maintained in the bacterial collection of the LaboREM-biotech, University of Sciences and Technologies of Bamako and (ii) select Bt strains carrying *cry* genes responsible of the synthesis of toxins (Cry1 and Cry2 proteins) active against Lepidoptera.

2. MATERIALS AND METHODS

2.1 Strains of *Bacillus thuringiensis* (Bt)

Bacillus thuringiensis (Bt) strains used in this study belong to collection of Laboratory of Research in Microbiology and Microbial Biotechnology (Laborem-Biotech) and that isolated from different ecological environment of Mali [20,21]. *B. thuringiensis* strains harboring cry1 and cry2 genes from bacterial collection of EMBRAPA Milho e Sorgo (Brazil), were also used as positive controls.

2.2 Determination of *cry* Gene Profile

We used polymerase chain reaction (PCR) to identify the cry gene content of native *B. thuringiensis* strains from microbial collection of LaboREM-Biotech in Mali. In total 53 native Bt strains producing parasporal inclusions were screened for two pairs of universal primers for the cry1 and cry2 genes as described by [22].

2.3 Identification of *cry* Type Genes

B. thuringiensis strains positive to *cry1* were subjected to further analysis for the *cry1* genotype. Gene-specific primers reported by [23,24]) were used to detect the presence of *cry1B, cry1C* and cry1F genes. Bacterial DNA was extracted and used as a template for PCR according to [15] and reference strains served as controls in PCR reaction. The cultures of 24 *B. thuringiensis* isolates with *cry1* gene were incubated overnight at 30℃ in LB broth medium with shaking. Five milliliters of LB medium was inoculated with 0.1 ml of the overnight culture and incubated at 30℃ for 16 hours with vigorous shaking.

Gene identification was performed by amplification of DNA of *B. thuringiensis* from Mali and reference strains, all from overnight grown cultures on agar plates. Sub-screening of 24 cry1-positive isolates was done with cry1 positive specific primers (*cry1F, cry1B* and *cry1C*) shown in above table 1. Final reaction volume was 20 µl, composed of: DNA 5 µl; 1.25X Buffer; 1,875 mM MgCl2; 0.5 mM dNTPs; 5pmol of each primer and 1 U/µl Taq DNA polymerase. Amplification was performed using a thermal cycler TECHNE (TC-3000). It was conducted a modified version of program described by [16] for *cry1F* genes *cry1B* and *cry1C* respectively as annealing temperature 58℃, 48℃ and 47℃. *Cry2* gene was amplified according to the program described by [3] with an annealing temperature at 55℃. A volume of 10 µl of each PCR product was subjected to electrophoresis on a 2% agarose gel for 1 h30mn at 100 V. The gel revelation was performed using ethidium bromide and the photography was performed using the E-Box VX2 system, Version 15.06. For the sub-screening Bt isolates from EMBRAPA were used as positive controls.

3. RESULTS

3.1 Determination of the *cry* Gene Profile

After amplification by the PCR, ten different profiles were observed and analyzed in Malian native *Bacillus thuringiensis* strains and some of them harbored the target *cry1* and *cry2* genes. Most of *Bacillus thuringiensis* strain identified by PCR carries at least one of these two *cry* genes (Fig. 1A and Fig. 2).

According to [25], we used the presence of more than one cry gene in some strains as a parameter to categorize the studied *B. thuringiensis* strains in three groups: (i) first group harboring only one cry gene (cry1): Ch0, ch1, ch2, ch3, Am1, Am2, DL0, S4', S5, CAA1, CAA2, I1, I3, I7', C1, C2, C3, C5, C7', Di1, Di2, Di3, Di3' C7 et Di4; (ii) second group constituted with strains harboring the two cry genes studied (*cry1* and *cry2):* S1, CAA3, I2, I4'', C4, Di5, D1P, D1G, D2P, D3P, D3G, D5, D6, D8, B1P, B1G, B2, B3, B4, B9P, B9G, DL1, DL2, DL3, and DL4; and (iii) third group composed with Bt strains which did not react with cry1 and cry2 specific primers: I4, C5 and N2.

3.2 Identification of *cry* Type Genes

Twenty four (24) out of 53 bacterial strains studied, were found to be positive for cry1 sub-type (Fig. 1) with potential toxicity to caterpillars were further characterized by PCR analysis using gene-secific primers for the cry1 genes: cry1F, cry1B and cr1C. Ten (10) strains showed presence of one single cry1 sub-type, thirteen (13) isolates exhibited amplification for two sub-types and only one (1) isolate exhibited amplification for the three targeted sub-types. I7', C1', C2 and S5 were positive for cry1F; I3 and N1 were positive for cry1B; Ch0 and Ch1 were positive for cry1C; CAA1, CAA2 and C3 were positive for cry1F+cry1B; C5', C7', Di2 and Di3 were positive for Cry1c+Cry1F; Ch2, Ch3, I1, Am1, Am2 and DL0 were positive for Cry1B+Cry1C and S4' was positive for the three

targeted cry1-sub groups (Cry1F, Cry1B and Cry1c). All targeted *cry1* sub-groups were amplified from the isolated strains (Fig. 2).

The different fragment and fragment sizes of cry and cry sub-genes determined by amplification were consigned in Table 2. The analysis of these data showed important size diversity in the different *cry* and *cry* sub-groups. We identified two (2) novel fragments for *cry1* F; four (4) for *cry1* B; six (6) for *cry1* C and five (5) for *cry2* (table 2). The presence of these new fragments suggests that these strains may contain potentially novel Cry proteins toxic to some important crop insect pests.

4. DISCUSSION

Native *Bacillus thuringiensis* strains from LaboREM-Biotech collection were characterized to identify *cry1* and *cry2* genes with four pairs of primers listed in Table 1. These primers were

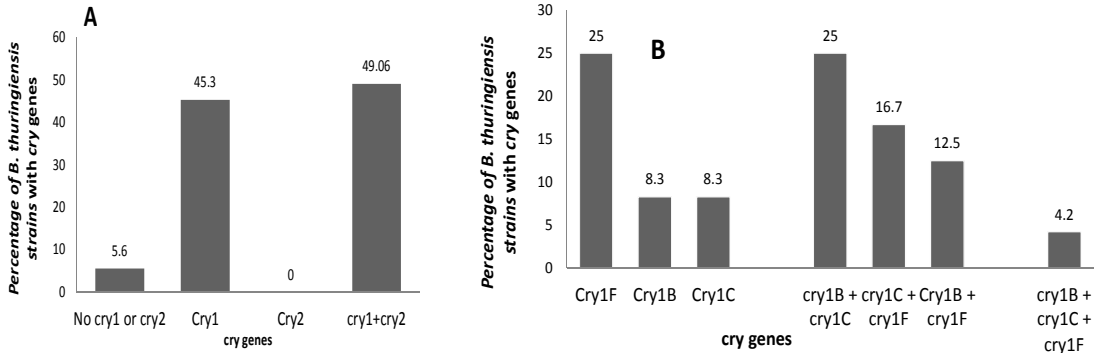

Fig. 1. Distribution of cry genes in the native *B. thuringiensis* strains from the LaboREM-Biotech bacterial collection in Mali. (A) Distribution of the single cry genes, (B) distribution of single and more than one cry1 sub-types

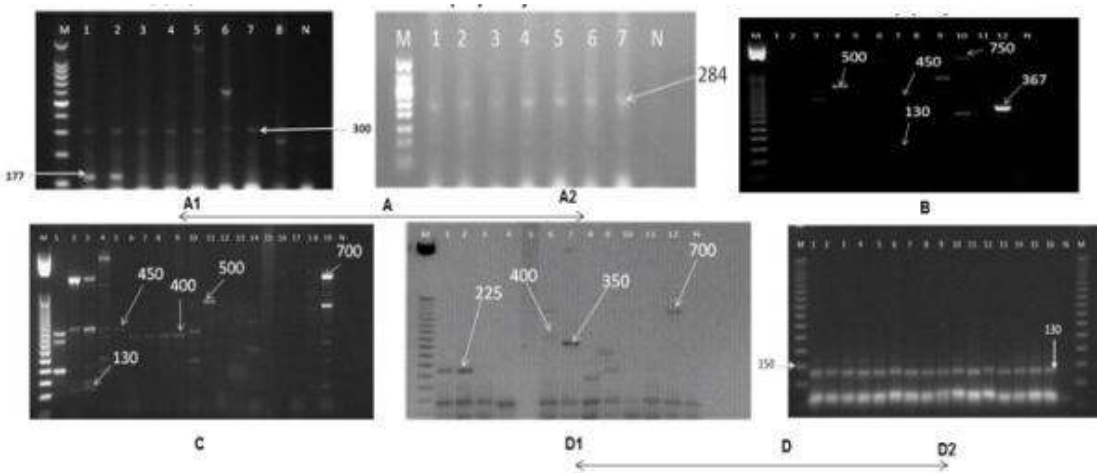

Fig. 2. PCR amplification products from local Bt isolates

***(A)** Fragments amplified with specific **cry1F** primers; A1: M = molecular marker (100bp GeLPilot) column 1-8 Bt strains: (B1g, B1p, D1g, D1p, D2p, B2, B3, D3p) and N: negative control, A2: M = molecular marker, column 1-7 Bt strains: (D3, D3', D4, D5, CAA1, CAA2 and CAA3) and N: negative control. (**B**) Fragments amplified with specific **cry1B** primers: M= molecular marker (50bp DNA Step Ladder). Column 1-12 Bt strains (Ch0, Ch1, Ch2, Ch3, DL0, DL1, DL2, DL3, DL4, Am1, Am2 and T06 and N: negative control. **(C)** Fragments amplified with specific cry1C primers: M: molecular marker (50bp DNA Step Ladder), column 1-19: Bt strains (S4, Ch0, Ch1, Ch2, Ch3, DL0, DL1, DL2, DL3, DL4, Am1, Am2, I1, I2, I3, I4, I4 ', T06 and HD-1) strains of Bt and N: negative control. (**D**) Fragments amplified with specific **cry2** primers: D1, M= molecular marker (50bp DNA Step Ladder). Column 1-12 Bt strains (S1, I4 '', N1, C4, I7, I2, D3, D2, F2, CAA3, D5, HD-125) **and D2,** M= molecular marker (50bp DNA Step Ladder). Column 1-16 Bt strains (D1P, D1G, D2P, D3P, D3G, D4, D5, D6, D8, B1P, B1G, B2, B3; B4; B9P and B9G and N: negative control)*

selected from highly conserved region among each group of gene. The determination of *cry* genes distribution and diversity in the *Bacillus thuringiensis* strains, isolated from soils of different areas of Mali, showed that, most of the Bt isolates (49,05%) in our bacterial collection harbor, in the same time, the cry1 and cry2 genes. According to [25] the *cry1* plus *cry2* genotype was the most abundant (40%) in native *Bacillus thuringiensis* isolated from fig tree environments in Turkey. It has been suggested by [26] that Bt strains with more than one cry gene, have high frequency genetic information exchange and may be toxic for several insect pests. We suggest that 49% of the Bt strains in our study can be considered as toxic to several insect pests and can be considered as potential biocontrol agents, active against caterpillars and African gall midge affecting seriously maize and rice crop in Africa, mainly in Mali [2]. In this study, 45.28% of the Bt strains in the collection harbor only one cry gene (cry1). According to [27] working on the distribution of cry-type genes in halophilc *Bacillus thuringiensis* isolates of Arabian Sea sedimentary rocks, cry1 genes were the most abundant in their collection (49.5%). [28], reported that the cry1 gene is the most abundant (73%) in native strains of *Bacillus thuringiensis* obtained from different ecosystems from Colombia. While, in this study, several Bt strains harbor more than one cry gene out of which the cry2 gene, no strain harboring only the *cry2* gene was obtained. Contrary to our result, [29] reported the presence of *cry1, cry2, cry3* genes. Among all the studied Bt strains, three isolates (5.66%) were considered to be negative for the four set of primers used in this study. Even that, these bacteria produce crystal inclusions [20,21] suggesting the presence of other Cry toxins. We think that these bacteria may have other cry genes not identified by the cry gene specific primers used in our study. Our results showed fragments sizes of 177; 284 and 300 bp for the *cry1*F. These last two fragments showed fragment sizes different to that of the pair of primers designed for *cry1*F sub-type and indicate the presence of new variants of the cry1F sub-type. These results are comparable to those found by Valicente and co-workers [15] who reported fragments sizes different to the expected ones (160 bp not 177 and 284 bp). Our results indicate that cry1F sub-type was the most frequent among native Bt strains in Mali. This result is different to that of Dos Santos and co-workers [30] who described a low frequency of this sub-type. Similarly to others [30,31] obtained a low *cry1*F gene frequency with 0.3% in strains of *Bacillus thuringiensis* isolated from soils in China; also a subtype frequency of 4.8% was obtained in strains isolated in Mexico [15]. Another research showed the absence of *cry1* F gene in strains of *B. thuringiensis* isolated in Thailand [32]. Contrarily, in brasilian isolates very high frequency fo cry1C and Cy1B (43.8 and 12.9%, resp.) was found while very low with 0.3% for *cry1*F [25]. Similarly Martınez et Caballero, working on the contents of *cry* genes and insecticidal toxicity of *Bacillus thuringiensis* strains from terrestrial and aquatic habitats, reported that cry1B gene was very frequent within thuringiensis strains from interrestrial and aquatic habitats [33].

Table 1. Characteristics of the specific primers

Gene	Sequence of primers	Anneling T°	Fragment (bp)	References
Cry1 F	F: 5'-GAGGATTCTCCAGTTTCTGC-3' R : 5'-CGGTTACCAGCCGTATTTCG-3'	58	177	[15]
Cry1 B	F : 5'-CTTCATCACGATGGAGTA A-3' R : 5'-CATAATTTGGTCGTTCTGTT-3'	48	367	[15]
Cry1 C	F : 5'-AAAGATCTGGAACACCTT T-3' R : 5'-CAAACTCTAAATCCTTTCAC-3'	47	130	[15]
Cry 2	F : 5'-GTTATTCTTAATGCAGATGAATGGG-3' R: 5'-CGGATAAAATAATCTGGGAAATAGT-3'	55	725	[23]

Table 2. Fragment sizes identified in *cry*1 and *cry2* genes

***cry* genes**	**Obtained size (bp)**							**Predicted size (bp)**
Cry 1F	**177 bp**	284 bp	300 bp					177
Cry 1B	130 bp	175 bp	**367 pb**	500 bp	750 bp			367
Cry 1C	**130 bp**	200 bp	350 bp	400 bp	450 bp	500 bp	700 bp	130
Cry 2	130 bp	225 bp	350 bp	400 bp	450 bp	**700 bp**		725

5. CONCLUSION

Strains of *Bacillus thuringiensis* isolated in Mali were identified by the PCR technique. Most of these strains harbor the *cry1* gene or the cry1 and *cry2* genes together. None of studied Bt strains harbored the cry2 gene only. The cry1 and cry2 genes were not detected in 3 Bt strains. In this study, the cry1F sub-type was found to be the most frequent. It will be interesting to know if both genes are expressed and efficient against simultaneously *Orseolia oryzivora* and *Helicoverpa armigera.* So, the next step will devoted to the investigation of protoxins from Bt strains isolated in Mali with the aim to develop efficient and low cost biopesticide to minize the negative impacts of insect pests on rice and maize production.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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