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Molecular characterization and insecticidal activities of Malian native crystalliferous *Bacillus thuringiensis*

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The objective of this work was to select the most active *Bacillus thuringiensis* (Bt) isolated from agricultural soils of Mali through the molecular characterization and the determination of insecticidal activities of the protein crystals, produced by these native isolates. Crystal proteins were extracted from *B. thuringiensis* culture, and characterized using the SDS-PAGE techniques. Their insecticidal activities were tested using third-instar larvae of *Helicoverpa armigera* in bioassay tests. The results showed that, of 62 *B. thuringiensis* treated, 52 isolates showed fragments varying between 10 and 140 kDa on 12% polyacrylamide gel. Cry1 and Cry2 protein crystals were recognized to be effective against Lepidoptera's larvae, which were found in 21% of the tested isolates. In addition to these two expected crystal protein weights, other molecular weights were observed at different proportions, suggesting the presence of other cry genes in the local *B. thuringiensis* isolates. Four native *B. thuringiensis* isolates were able to kill 95 to 100% of *H. armigera* 3rd-instar larvae. Only one native of *B. thuringiensis* isolate was able to kill 100% of the *H. armigera* larvae. This is the first study for molecular characterization of Malian native *B. thuringiensis* isolates, showing the efficacy of the native *B. thuringiensis* against an important agricultural insect pest.

Key words: Protein crystals, *Bacillus thuringiensis*, cry genes, insecticidal activity, molecular characterization, *Helicoverpa armigera*, Mali.

INTRODUCTION

The introduction and subsequent proliferation of synthetic insecticides has played a key role in increasing agricultural productivity, protecting forest crops, and controlling insect vectors of human diseases (Joung and Côté, 2000). In Mali, the damage caused by insect pests can be as high as 60% crop loss (Hamadoun, 1996). The use of synthetic insecticides has led to the emergence of resistant biotypes that are no longer controlled by major groups of

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chemical insecticides (Pooja et al., 2013).

A promising alternative to synthetic insecticides that has attracted attention is the development of protein toxins, produced by *Bacillus thuringiensis* (Bt) as insecticides (Pooja et al., 2013). *B. thuringiensis* is a sporoform ubiquitous gram-positive bacterium that forms protein crystals (protoxins) during the stationary phase of its growth cycle (Schnepf et al., 1998). These protein exhibit toxious activities against insect larvae (Nazarian et al., 2009; Pardo-Lopéz et al., 2013). *B. thuringiensis* toxins are specific to a limited number of insect species without any toxicity to humans or other organisms (Bravo et al., 2011). Some of them are toxic to a large number of insect species in this order; Lepidoptera, Diptera and Coleoptera in addition to some Homoptera (MacIntosh et al., 1990; Bravo et al., 2007; Porcar et al., 2009; Palma et al., 2014), and Nematodes (Wei et al., 2003).

PCR is a rapid tool for preliminary characterization of *B. thuringiensis*. The reliability of predicting insecticidal activity on the basis of PCR results is dependent on the expression of cry genes. The cry genes that lack promoters or functional genes, which are weakly expressed, will produce an erroneous prediction. So, for a better characterization of Bt strains, essential complements to the identification of cry genes, should be made through the determination of parasporal crystals composition, by polyacrylamide gel electrophoresis using sodium dodecyl sulfate (SDS-PAGE) and biological activity assays (Porcar and Juarez-Perez, 2003). In Mali, over the last three years, there has been an intense interest in the collection and analysis of local Bt strains from various environmental samples in Mali (Kassogué et al., 2015). The aim of this work was to characterize and describe the toxicity of several *B. thuringiensis* toxins (Cry1B, Cry1C, Cry1F and Cry2) for *Helicoverpa armigera* and *Orselia oryzivora*.

MATERIALS AND METHOD

B. thuringiensis strains

Fifty-two (52) *B. thuringiensis* strains isolated from soil and plants of Mali, harboring cry1 and/or cry2 genes (Fané et al., 2015) were analyzed by SDS-PAGE for cry crystal proteins production and distribution.

Production, extraction and separation of crystalline proteins

To produce crystal proteins, *B. thuringiensis* strains were cultured on Luria Bertani (LB) solid medium enriched with salts according to the method described by Valicente et al. (2010), for 24 h at 30°C. From the 24 h culture, the strains were transferred in 50 ml nutrient broth and incubated at 30°C for 72 h with continuous stirring at 200 rpm (Ammouneh et al., 2011).

Proteins were extracted using the modified protocol of Ammouneh et al. (2011). To do this, the suspension obtained was incubated in ice for 20 min and then centrifuged at 2000 *g* for 5 min using a centrifuge (Sigma 2-6 E). 2 ml of the pellet were taken and incubated in an ice for 15 min and then centrifuged with a centrifuge (Eppendorf 5424) at 14500 rpm for 10 min. The pellet obtained was resuspended in 1 ml of 0.5 M iced NaCl and then centrifuged at 14,500 rpm for 5 min. These pellet was resuspended in 1 ml of solution (1% SDS and 0.01% beta-mercaptoethanol) and the mixture was boiled at 95°C for 10 min, and centrifuged at 14500 rpm for 10 min. The supernatant was harvested and analyzed using 12% SDS-PAGE as described by Laemmli (1970). Electrophoresis was performed in a system (Omni PAGE 'WAVE' Electrophoresis Systems) at 90 V and 45 mA for 16 h. The gel was stained with 0.1% coomassie blue R250. Protein masses were determined by comparison with a broad range protein molecular weight marker.

Insect and bioassay

Several larvae of various instars of about 100 *H. armigera* were collected from maize and tomato fields in Mali, July, 2016 and were used for mass rearing under controlled conditions on an artificial diet (Abbasi et al., 2007), in a growth chamber at 25°C by using a relative humidity of 70% in a photoperiod consisting of 16 h of light and 8 h of darkness.

The population was reared for three generations in the laboratory before insects, which were used for bioassays (Fiuza et al., 1996).To determine the insecticidal activity of the spore-crystal mixtures, different *B. thuringiensis* isolates were screened on third-instar larvae of *H. armigera* as described by Aboussaid et al. (2009). In the first screening, the bioassay was performed with non-concentrated spore-crystal suspensions. For each isolate, 30 larvae were used. Mortality was observed after 3 days.

Bioassay was replicated with the isolates showing mortality rate, superior to 30%. For the chosen strains, the spore-crystal suspensions were centrifuged for 15 min at 15 000 *g* and 4°C and the spores and crystals were collected in the pellets. The pellets obtained were washed three times with phosphate buffered saline (PBS) containing 0.005% Triton X-100 in order to promote the bursting of the bacterial cells. The pellets were lyophilized and used to determine their insecticidal activity (Weathersbee et al., 2006). Three dilutions were used for each isolate and 30 third-instar larvae were used for each dilution. Mortality was observed after 3 days as described by Hassani and Gaouar (2008).

RESULTS AND DISCUSSION

Profile and frequency of Cry proteins synthetized by the native *B. thuringiensis* isolates

To determine the ability of the Malian *B. thuringiensis* (Bt) isolates to synthetize Cry proteins (crystal proteins), the 62 isolates were characterized by SDS-PAGE. The results obtained indicate that, some isolates were able to synthesize Cry proteins with molecular weights of about 130 and 70 kDa (Figure 1).

The protein profiles of cry1 B, cry1C, cry1F and cry2 genes observed on the 12% polyacrylamide gel (SDS-PAGE) showed bands of molecular weight between 10 and 140 kDa (Table 1). These results are in agreement with those of Gao et al. (2008) who observed parasporal crystals in 342 *B. thuringiensis* isolates, composed of more than one protein with molecular weights ranging between 28 to 150 kDa which is usually 65 to 140 kDa, with seven isolates producing a single protein Crystal of 50 kDa.

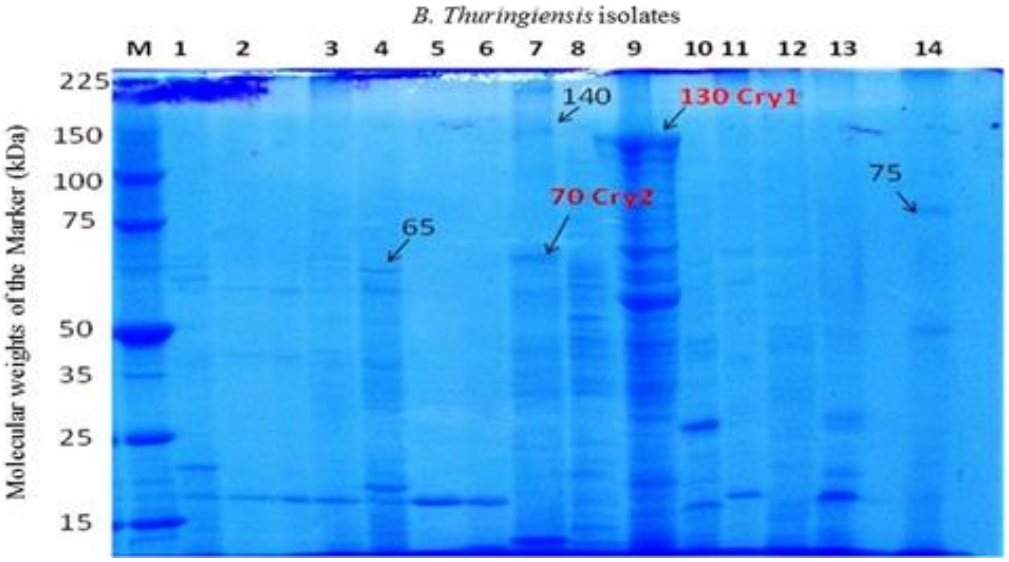

Figure 1. SDS-PAGE of the mixed spore-crystals of the *Bacillus thuringiensis* isolates. M, marker of molecular weight in KDa (broad range protein molecular weight marker); column 1 to 14 strains of Bt (D5, D8, D1G, B1G, B5, C4, I4 '', I4'N, I7, Dr2, DL1, DL2, DL4 and DL5).

Table 1. Molecular weight, number and frequency of protein bands.

Molecular weight (kDa)	Bands	
	Number	Frequency of bands (%)
140	3	6
125	5	10
130-70	11	21
100-110	5	10
80-90	4	8
66-68	5	10
60-65	13	25
50-55	14	27
40	6	10
20-25	5	10
10-17	4	8

B. thuringiensis showed bands with approximately 130 and 70 kDa which were expected in protein sizes as expected during the present study (Table 2). These results can be compared with those of Ammouneh et al. (2011) who obtained protein sizes of about 130 and 65 kDa in the local strains of *B. thuringiensis* of Syria. Similarly, dos Santos et al. (2009) found the presence of two main polypeptides of approximately 130 and 65 kDa of the proteins obtained from the spore-crystal mixtures by SDS-PAGE. Apart from these two sizes, 130 and 70 kDa and other protein molecular masses were observed in several Malian local strains which would indicate the presence of other Cry genes. The results obtained by Fiuza et al. (2012) are different from those obtained by the present investigation because they showed that Cry9 proteins have about 130 kDa.

In addition to these two expected crystal protein weights, other molecular weights were observed at different proportions (Table 1), suggesting the presence of other cry genes in the local Bt isolates analyzed. This result is in agreement with those obtained by Pinheiro (2013), who showed that most of the *B. thuringiensis* analyzed are capable of producing more than one type of crystal. Gonzalez and Carlton (1984) and Lereclus et al. (1989) showed that, crystals can be formed by different Cry and / or Cyt proteins as occurs for example in *B. thuringiensis* sub sp. *Israel* which has 5 genes encoding Cry proteins of cytolysin located on the same plasmid of 72MDa.

Among the *B. thuringiensis* isolates analyzed, 21% possessed the desired band sizes showing the expression of cry1 and cry2 genes which were harbored by these bacteria (Table 1 and 2). In 50% and more of the isolates analyzed, cry1 and cry2 genes were not expressed as shown by the absence of Cry endotoxins (Table 3). In the isolates harboring, only a cry gene and the cry2 gene expresses more than the cry1 (50% for cry2 and 38.46% for cry1), the same phenomenon is observed in the isolates, harboring at the same time cry1 and cry2 genes (27.58% for cry2 and 3.44% for cry1) (Table 3). Contrary to our results showing more than 50% of non-expressed cry genes, Ammouneh et al. (2011) supported the idea of expression of the cry1 and cry2 genes in all their *B. thuringiensis* isolated from Syria.

Table 2. Isolates of *Bacillus thuringiensis*, *cry* gene(s) harbored by each isolate, expected and non-expected Delta-endotoxins sizes (kDa) obtained.

***B. thuringiensis* isolates**	***Cry* genes**	**Size of Delta-endotoxins (KDa)**	
		Expected	**Not expected**
DL1	*cry1* B, *cry1* C, *cry2*	70*	-
Dr2	*cry1* F, *cry1*C	130**, 70	65, 60, 50
Ch2	*cry1* B, *cry1* C, c*ry2*	130, 70	-
S1	*cry1* B, c*ry*1F, *cry* 2	130,75*	60, 25 10
Dr3'	*cry1* F	130	50, 40, 25
D3P	*cry1* F, *cry2*	-	40
D5	*cry1* F, *cry2*	70	20, 17
DL2	*cry1* B, *cry1*C, *cry2*	140**, 130, 70	-
Ch1	*cry1F, cry2*	130, 70	-
C5	*cry1* F	130	-
I7'	*cry1* F	-	100, 50
D2P	*cry1* F, *cry2*	-	65, 60
C4	*cry1* F, *cry1* B, *cry2*	-	125, 10
B4	*cry2*	-	60
I2	*cry1* C, *cry2*	70,75	125, 90

*CryII delta-endotoxin; **CryI delta-endotoxin.

Table 3. Percentage of Cry Delta-endotoxins (crystal proteins) produced by *Bacillus thuringiensis* harboring different cry genes.

Bt with cry genes	**Cry delta-endotoxins (%)**				
	Total	**Cry1**	**Cry2**	**Cry1+cry2**	**No Cry endotoxin**
cry1+cry2	29	3.44	27.58	17.24	51.72
cry1	13	38.46	-	-	61.54
cry2	2	-	50	-	50

Insecticidal activity of the *B. thuringiensis* isolates on *H. armigera* larvae

Morphology and distribution of the B. thuringiensis crystal (Cry) proteins

Microscopic observation of bacterial smears colored with coomassie blue, at 1000 magnification using oil immersion, made it easy to distinguish the spores from the crystals which will appear in blue. Thirty-five (35) of the *B. thuringiensis* isolates studied, produced crystals (Delta (δ)-endotoxins). Three types of crystals (Bipyramidal, spherical and cubic) were found alone or in combination (Table 4). The types of crystals, the number and identity of the strains producing these crystals as well as the numbers and percentages of these strains are presented in Table 4.

Analysis of the data in this table shows that, only DL4 and D8 isolates produce bipyramidal crystals alone. *B. thuringiensis* isolates producing spherical crystals alone or combined with bipyramidal crystals represent respectively, 22.85 and 25.71% of the crystal-producing *B. thuringiensis* isolates. The isolates producing Cry1 and Cry2 proteins as mixture of spherical-cubic crystals represent 20% of the total crystal-producing bacteria. *B. thuringiensis* isolates producing cubic crystals alone (14.28%) and others producing a mixture of bipyramidal-cubic crystals (11.42%) were also identified.

Mortality rate of H. armigera larvae caused by the B. thuringiensis crystal proteins

The bioassay tests were carried out on a total of 30 larvae per isolate. Data of insecticidal activity of the tested *B. thuringiensis* isolates on *H. armigera* larvae are presented in Table 5. Of the isolates, producing Cry1 and/or Cry2 proteins tested 47% had a mortality rate greater than 80% while 35.30% had a mortality rate between 80 and 75%, and 17.64% of the isolates killed

Table 4. Morphology of crystals, identity and percentages of the *B. thuringiensis* isolates producing these crystals.

Crystals	Morphology of the Delta-endotoxin crystals					
	Spherical	**Bipyramidal**	**Cubic**	**Spherical-Bipyramidal**	**Spherical- cubic**	**Bipyramidal-Cubic**
***B. thuringiensis* isolates**	DL5	DL4	C3’	Dr2	D1P	I2
	I7	D8	S1	DL1	DL5’	C5’
	CH3	-	I4″	DL2	B1G	D1G
	B1P	-	CH1	AM2’	C5	D11
	Dr3	-	C3″	I7’	Dr5	-
	D3P	-	-	D5	D1P	-
	AM1	-	-	B4	B5	-
	DL3	-	-	D2P	-	-
	-	-	-	D3G	-	-
Total	**22.85**	**5.71**	**14.29**	**25.71**	**20**	**11.43**

Table 5. Efficacy of *B. thuringiensis* isolates as expressed by the mortality rate of *Helicoverpa armigera* larvae (%).

	Mortality rate of *Helicoverpa armigera* larvae (%)									
	0	100	95	90	85	80	75	70	60	55
Isolates	Control	DL_1	Dr_2 L_2 CH_2	S_1	$Dr_{3'}$ D_3P D_5	DL_2 CH_1 D_1P	C_5 I_7’ D_2P	C_4	B_4	I_2

Figure 2. Insecticidal activity of *B. thuringiensis* isolate Dily1 against *H. armigera* larvae.

between 70 and 55% of *H. armigera* larvae (Table 5). As observed in this work, Bravo et al. (1998) and Crickmore et al. (1998) indicate that, the most known proteins on Lepidoptera are encoded by the Cry1, Cry2, and Cry9 genes.

B. thuringiensis producing spherical (D3P), or spherical combined to other Cry endotoxins morphology (DL1, D5…) were identified as more actives. The results showed an efficacy ranging from 55 to 100%, obtained with the isolate Dily1 (Figure 2). These results are clearly superior to those of Lalitha et al. (2012) who obtained a Bt mortality rate on *H. armigera* in stage 2, ranging from

5.56 to 94.4%.

Conclusion

Fifty-two (52) isolates showed fragments varying between 10 and 140 kDa on 12% polyacrylamide gel. Cry1 and Cry2 protein crystals recognized to be effective against Lepidoptera when found in 21% of the isolates.

Out of the 52 Bt isolates tested, 4 were able to kill 95 to 100% of *H. armigera* third-instar larvae. Only one native Bt isolate was able to kill all the *H. armigera* larvae. This study constitutes to our knowledge, the first molecular characterization of Malian native of *B. thuringiensis* isolates, showing the efficacy of the native Bt isolates against the important agricultural insect larvae.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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