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Aphicidal activities of Moroccan *Bacillus thuringiensis* strains against cotton aphid (*Aphis gossypii*)

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Abstract: The objective of this study was to evaluate the insecticidal effect of toxins from Moroccan *Bacillus thuringiensis* strains (Berliner) (Bt) on *Aphis gossypii (*Homoptera: Aphididae). Aphis gossypii is one of the most pests of Moroccan crops. Their management is based traditionally on using chemical products. Some of them are well known to be potentially toxic to the environment and human health. Therefore, alternative strategies for aphid management in crops have been developed in recent years, including a biological control using toxins of bacterial strains. In this study, the artificial diet bioassay was used to screen the aphicidal effect of 82 Bt toxins against first instar nymphs and third instar nymphs of *A. gossypii*. Among the examined Bt strains, eleven showed a high insecticide activity against *A. gossypii* stages. In addition, the assessment of the lethal concentration (LC_{50}) of selected Bt revealed that the local BtA4, BtA1 and Bt21.6 exhibited higher insecticidal activity against first instar nymphs of *A. gossypii* (LC_{50} (BtA4)=0.15, LC_{50} (BtA1)=0.23 and LC_{50} (Bt21.6)=0.25 mg/ml) and the selected strains BtB6, BtA10 and Bt21.6 exhibited the relatively best activity third instar nymphs of *A. gossypii* (LC_{50} (BtB6)= 0.48, LC_{50} (BtA10)= 0.79 and LC_{50} (Bt21.6)= 1.14 mg/ml) of *A. gossypii*. Therefore, the results of this study indicate that the selected *B. thuringiensis* strains have great potential to be used in the integrated *A. gossypii* management.

Keywords: *Bacillus thuringiensis*; *Aphis gossypii*; artificial diet bioassay; biotoxicity; LC_{50}.

1. Introduction

Aphis gossypii is one of the serious pests worldwide, infesting more than 500 plant species belonging to different families such as Cucurbitaceae, Solanaceae, Brassicaceae, Asteraceae, Malvaceae, Rosaceae, Amaranthaceae, among others [1, 2]. In Morocco, agricultural producers have encountered severe aphid problems despite the continuous applications of pesticides [3]. Aphids have a serious economic effect because of their pervasive proliferation due to their biological characteristics, specific polymorphism, and other alternating kinds of reproduction.

Aphis gossypii feeds on leaves causing damage to chlorophyll. In addition, they produce honeydew which allows sooty molds to grow, resulting in a decrease in fruit quantity and

quality. To control *A. gossypii*, synthetic pesticides have been used by farmers. But the overuse of these chemical products during the past several years has caused many problems such as environmental pollution, harmful to beneficial insects, accumulation of toxicity at different trophic levels, and the emergence of resistance to pesticides [4, 5]. Therefore, a need to develop alternative strategies such as entomopathogenic bacterial toxins: *Bacillus thuringiensis* (*Bt*) has become necessary.

Bacillus thuringiensis is an aerobic Gram-positive bacterium. During sporulation, *Bt* produces the proteinaceous parasporal crystals [6, 7]. These crystals consist of two types of proteins named Crystal (*cry*) and cytolytic (*cyt*) proteins [8], which are known for their insecticidal proprieties [9]. Proteins Cry has shown a high specificity to target insects and has been considered safe toward non-target organisms, plants, and humans, and completely biodegradable [10–13]. Therefore, *Bt* is one of the most effective biopesticides for insect control and represents roughly 2% of the total insecticide sales [14].

Several studies have shown *Bt* to be highly toxic against insects and mites of different orders [15–20]. In previous studies carried out in our laboratories, the *Bt* strains isolated from five different areas that *Argania spinosa* tree grows in Morocco showed a high insecticidal effect on *Ceratitis capitata* stages [21–23] and against *Eutetranychus orientalis* adults [17]. Thus, during the last years, there has been an important improvement in the screening of *Bt* collections to get isolation useful for pest control [24, 25]. Many aphid species have been found to be most vulnerable to *Bt*; such as the pea aphid *Acyrthosiphon pisum* [26], the potato aphid, *Macrosiphum euphorbiae* [15], the grain aphid *Sitobion avenae* [27], the green-peach aphid, *Myzus persica*e [28, 29] and the cotton aphid *Aphis gossypii* [30].

To our knowledge, there is no available study about the insecticidal effect of Moroccan *Bt* strains, especially those isolated from the endemic Argan forest on *A. gossypii*. Hence, this study aims to assess the toxicity effects of *Bt* strains, isolated from Argan regions in Morocco against *A. gossypii* stages, using artificial diet bioassay under laboratory conditions.

2. Materials and Methods

2.1. Rearing of A. gossypii.

A colony of *A. gossypii* was obtained from the Insectarium of Agafay orchards, Marrakech, Morocco (N31°30'04.0", W8°14'54.4"). The insect colonies had been reared for several generations at Agafay Insectarium. This aphid species were continuously reared on *Phaseolus vulgaris* L. under controlled conditions at 23± 2°C, 60 ± 5% R.H., and with a 16: 8 h (L:D) photoperiod.

2.2. Production of Bt toxins.

A loopful of bacteria taken from a colony of 82 strains grown in medium CCY agar was used to inoculate a tube with 4.5 ml of medium CCY liquid (pre-culture) and then placed at 28℃ during 48 h with 200 rpm of agitation to grow. To verify the formation of the toxins (over 90% sporulation is optimum), an aliquot was taken from the pre-culture and observed under phase-contrast microscopy (DM2500, Leica Microsystems, Germany). The pre-culture was heated at 70℃ for 20 minutes to eliminate the vegetative cells. 40 ml of the main culture was inoculated with 1/1,000 volumes of synchronized pre-culture and incubated as mentioned above. Then the whole culture was centrifuged for 10 minutes at 9,000 ×g.

The supernatant was discarded, and the pellet was washed once with ice-cold 1 mol/l NaCl and 10 Mmol/l EDTA solutions. Then the pellet was concentrated by lyophilization to express the *Bt* toxins in mg and stored at -20 °C until bioassays. All steps have been done on ice to limit the proteolysis after centrifugation.

2.3. Selection of active bacterial strains.

In order to select the *Bt* strains with high toxicity effects against *A. gossypii* nymphs. We conducted preliminary dose setting experiments with first and third instar nymphs of A. gossypii using an artificial diet [29]. This diet contained 500 mM sucrose (pH: 7.5-8), 150 mM amino acids, minerals, and vitamins, and different dilutions of toxins. The *Bt* strains that showed a high percentage of mortality, over 75%, were selected to be used in the experiment. Based on the preliminary trial, eleven *Bt* strains were selected between eighty tows (Table 1). Five concentrations (0 (control), 0.1, 0.5, 1, 2, and 3 mg/ml) were chosen to be used in the experiment, and 150 µl of each concentration was mixed with the artificial diet.

Table 1. Morphological characterization (phase-contrast microscopy), biochemical (SDS-PAGE), and genetic (SDS-PAGE and PCR) of the 11 Moroccan strains of the selected *Bt*s.

N°	Strains	Crystal form	Gene cry (PCR)
1	Bt A1	*Spherical*	*cry7/8*
2	Bt A4	*Irregular*	*cry7/8+cry9*
3	Bt A10	*Spherical*	*cry4+cry7/8*
4	Bt A14	*Spherical*	*Cry 11*
5	Bt A-Mg Mg2.7	*Spherical*	*Cry 11*
6	Bt B6	*Spherical*	*cry7/8+cry9*
7	Bt21.6	*Irregular*	*cry4+cry11+cyt1*
8	Bt 9	*Irregular*	*cry 4*
9	Bt 32.2	*Crystal>1*	*NI**
10	Bt 26.4	*Spherical*	*cry 4*
11	Bt 32.3	*Irregular*	*cry 9*

***NI: No Identified.**

2.4. Bioassay.

The feeding membrane assays were used to assess the toxicity of selective strains of Bt against first and third instar nymphs of A. gossypii. Different concentrations of toxins of each strain were mixed with the artificial. No toxins were added to the control. The diet was presented to aphids in stretched parafilm sachets containing 0.4 ml of artificial diet. Plastic tubes opening at both ends (cylindrical plastic tubes with 5 cm diameter and 4 cm height) were used as a test chamber (Figure 1). The diet in the form of stretched parafilm sachet was deposited into the test chamber. The other end was covered with a muslin cloth with fine mesh. From this end, 20 first instar nymphs and the third instar nymphs of *A. gossypii* were introduced. The concentration of *Bt* toxins was 3, 2, 1, 0.5 and 0.1 mg/ml. Each concentration was replicated five times for each strain. The sterile distilled water was used as a negative control and the commercial Bt strain BT-M as a positive control. The bioassays were conducted under laboratory conditions at 23± 2°C, 60 ± 5% R.H., and a 16: 8 h (L:D) photoperiod. Aphid stage mortality was recorded after 3 days of treatment.

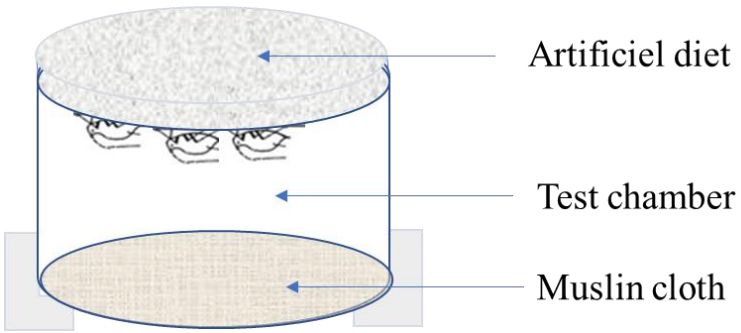

Figure 1. Test chamber: a plastic cylinder with 5 cm diameter and 4 cm height, with a mesh on the bottom and the top open. On this open side, the diet sachet was introduced.

2.5. Statistical Analysis.

The lethal concentrations' values (LC_{50} and LC_{90}) were calculated by using probit analysis SPSS 25.0 Statistical Software. The confidence interval of estimate LC values was 95%, and when the LC values did not overlap, they were considered significantly different.

3. Results

3.1. Insect bioassay.

Among the 82 *Bt* strains evaluated against first instar nymphs of *A. gossypii* through the selective bioassays, only fifteen strains showed insecticidal mortality greater than 75%, corresponding to 13.41% of the strains (Table 2). Most of the strains (42.68%) exhibited very low toxicity (0-25% mortality) against *A. gossypii* nymphs, 29.28% of the strains had low toxicity (25.1-50% mortality), and 14.63% of the strains were moderately toxic, causing mortality from 50.1 to 75% mortality (Table 2).

Table 2. Groups of *Bt* strains based on their toxicity against *A. gossypii* stages.

Group	Toxicity against *A. gossypii* in % mortality	Number of strains	% of strains
I	0-25	35	42.68
II	25.1-50	24	29.28
III	50.1-75	8	14.63
IV	75-100	15	13.41

3.2. Toxicity effect of Bt strains against first instar nymphs.

The insecticidal activities of selected *Bt* strain against first instar nymphs of *A. gossypii* are presented in Table 3. The control did not show any nymph mortality. The nymphicidal activity varied with tested *Bt* strains. Among the selected strains BtA4, BtA1 and Bt21.6 exhibited the highest potency with LC_{50} values of 0.15, 0.23 and 0.25 mg/ml and LC_{90} values of 0.53, 0.73 and 0.89 mg/ml, respectively. The lowest efficacy was observed for Bt26.4, BtA14, and BtB9 with LC_{50} values of 2.94, 3.19 and 3.94 mg/ml and LC_{90} values of 7.41, 9.65 and 10.90 mg/ml. The five remaining *Bt* strains show intermediate nymphicidal activity. The LC_{50} values of these strains were statistically similar to that of the strain Bt-M that showed LC_{50} of 1.37 mg/ml.

Table 3. LC_{50} and LC_{90} values of the selected *Bt* strains against first instar nymphs of *A. gossypii* after 3 days of bioassay.

Strains	LC_{50} (95% CI) (mg/ml)	LC_{90} (95% CI) (mg/ml)	Slope ± SE	$\chi 2$(df=4)
Bt A1	0.23 (0.15-1.29)	0.73 (0.37-3.02)	1.53±0.19	3.65
Bt A4	0.15 (0.11-0.93)	0.53 (0.29-2.63)	2.72±0.14	11.82
Bt A10	1.21 (1.03-1.48)	3.27 (2.36-4.09)	1.81±0.09	8.01
Bt A14	3.19 (3.00-4.38)	9.65 (6.48-13.02)	2.13±0.61	9.32
Bt A-Mg Mg2.7	2.21 (1.89-3.76)	6.28 (4.60-9.73)	3.47±0.32	3.95
Bt B6	0.85 (0.23-1.49)	2.07 (0.87-4.42)	1.21±0.09	21.55
Bt21.6	0.25 (0.16-1.76)	0.89 (0.75-3.74)	1.13±0.91	4.25
Bt B9	3.94 (2.02-5.07)	10.90 (4.83-13.03)	2.03±0.25	2.16
Bt32.2	1.39 (1.16-1.76)	3.88 (3.12-6.59)	1.39±0.26	7.49
Bt26.4	2.94 (1.52-7.17)	7.41 (4.47-18.58)	2.56±1.92	10.21
Bt 32.3	1.58 (1.18-4.72)	3.55 (2.53-6.81)	1.84±0.52	6.15
Bt-M	1.37 (1.02-2.97)	4.27 (3.44-6.18)	4.46±2.12	13.07

3.3. Toxicity effect of Bt strains against third instar nymphs.

The data shown in Table 4 revealed that all tested *Bt* strains showed an interesting nymphicidal potency against the third instar nymphs of *A. gossypii*, whereas in the controls, any mortality did not observe. The strains Bt B6, Bt A10 and Bt21.6 showed the highest nymphicidal activity with LC_{50} values of 0.16, 0.31 and 0.36 mg/ml and LC_{90} values of 0.48, 0.79 and 1.14 mg/ml. Intermediate LC_{50} values of 0.84, 1.27 and 1.34 mg/ml were recorded for Bt A4, Bt A1 and Bt32.2, respectively. The LC_{50} values of these strains were statistically similar to the strain Bt-M that showed LC_{50} of 0.95 mg/ml. The five remaining *Bt* strains show the lowest nymphicidal potency.

Table 4. LC_{50} and LC_{90} values of the selected *Bt* strains against third instar nymphs of *A. gossypii* after 3 days of bioassay.

Strains	LC_{50} (95% CI) (mg/ml)	LC_{90} (95% CI) (mg/ml)	Slope ± SE	$\chi 2$(df=4)
BtA1	1.27 (1.02-1.41)	3.48 (2.21-4.42)	2.37±0.17	3.26
BtA4	0.84 (0.29-1.11)	2.78 (0.99-4.21)	3.42±1.92	11.61
BtA10	0.31 (0.19-1.12)	0.79 (0.32-3.15)	1.32±0.45	8.27
BtA14	3.23 (3.16-5.34)	9.58 (9.08-12.55)	2.63±1.01	2.01
BtA-Mg Mg2.7	2.08 (1.78-3.24)	5.39 (4.41-8.91)	1.82±0.78	2.19
BtB6	0.16 (0.12-0.29)	0.48 (0.83-2.31)	2.31±0.24	17.82
Bt21.6	0.36	1.14	2.51±0.90	3.19
BtB9	3.72 (1.68-6.21)	7.48 (2.97-9.48)	1.91±1.05	9.81
Bt32.2	1.34 (0.99-2.10)	4.26 (2.41-7.18)	1.65±0.61	16.05
Bt26.4	2.32	5.67	0.95±0.05	5.14
Bt32.3	1.80 (1.11-3.05)	5.42 (2.98-11.24)	1.43±0.11	19.13
Bt-M	0.95 (0.64-2.03)	2.52 (1.89-4.21)	2.01±0.59	9.22

4. Discussion

Actually, many control methods, including chemical insecticides, biological control, *Bt* crops, and cultural practices, have been used in integrated aphid management to achieve more effective crop protection [31-34]. The environmental, social, and economic benefits offered by the *Bt* bioinsecticides and insect-resistant *Bt* plants have driven increasing adoption of these management approaches for managing insect pests belonging to divers' orders of [35–38]. However, pest populations can develop resistance to several types of *Bt* pesticide proteins, whether under open field or controlled conditions, reducing bioinsecticides and transgenic plants [39]. This scenario encourages the research for novel *Bt* strains and genes that can be used in integrated pest management strategies.

In the current study, the aphicides activities of *Bt* strains were evaluated to discover a promising strain to be used in developing biopesticides against *A. gossypii*. We found that

18.29% of studied strains were highly toxic against 1st-instar and 3rd instar nymphs of *A. gossypii*. In addition, among the selected strains, BtA4, BtA1, and Bt21.6 displayed the highest toxicity against the 1st-instar, while the selected strains BtB6, BtA10, and Bt21.6 showed the high nymphicidal potency on 3rd instar of *A. gossypii* as they had displayed the lowest $LC_{50,90}$ values. Some studies have shown that the proportion of strains toxic to different orders of insect stages is often low, as shown in our study [22, 40]. Our result showed that the toxicity of selected strains varied considerably towards the *A. gossypii* stages. The strain Bt21.6 caused a high level of mortality to both stages of aphid, whereas strain Bt B9 had low toxicity on aphid stages. Furthermore, the strain BtA4 was effective on 1st-instar, while the strain BtA10 had high toxicity towards the 3rd instar of *A. gossypii*. Our study showed that some *Bt* strains had a wide range of activity against both stages of *A. gossypii*, other strains were more specific, showing that the screening of the insecticidal effect of *the* Bt strains against insect pests under controlled conditions is a crucial step to discover the strains that had a great efficiency and to evaluate their feasibility to be used as biopesticides.

The difference in susceptibility of *A. gossypii* stages on the selected Bt strains in the current study may be explained by the genes that coding for aphicidal activity against the two stages of the aphid could be different [41, 22]. The chemical composition of the gut of the two stages of aphid could be affecting the solubilization and proteolytic processing of Bt proteins, therefore the activation or inactivation of the protoxin [42, 43]. In addition, other factors can influence the effectiveness of *Bt* strain, such as the antagonistic or synergistic interactions between *Bt* proteins (Cry and Vip) [44].

Many research on the efficacy of *Bt* that have indicated an insecticidal activity against aphids and other Homoptera insects. In fact, Torres-Quintero et *al.* [45] and Palma et al. [28] confirm that Bt strains tested in the bioassays are responsible for the mortality of *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) after being fed with different toxins of *B. thuringiensis* strains. However, it is interesting to point out that other studies have shown contrasting results. Macintosh et *al.* [46] showed that the green-peach aphid was insensitive to many *Bt* toxins. Furthermore, Oatman [47] reported that *Bt* toxins were not inefficient in controlling Apple Aphid. The difference observed between the studies mentioned above and our results could be related to two eventual factors: the types of the Cry proteins produced by *Bt* strains or the possibility of the presence of other genes not identified. Further studies are needed to discover all *cry* genes that may contribute to these studied strains' toxicity.

Bt strains with diverse genes might synthesize many Cry and Vip proteins, which may have increased toxicity and target a wider range of pests [48]. In fact, the presence of many cry genes allowed the variation of active modes of synthesized Cry proteins that may decrease the risk of development of resistance in the target pest populations [49, 50]. In concordance with this, our results showed that the five studied BtB6, BtA10, BtA4, BtA1, and Bt21.6 were the most toxic to studied aphid stages and these *Bt* strains showed a high diversity of cry genes. Nonetheless, our results suggest that the selected strains may be a potential source of genes for the development of insect-resistant transgenic crops.

5. Conclusions

The current work identified *Bt* strains that have shown a great potential to be used in the formulation of biopesticides for integrated aphid management. In addition, the selected *Bt* strains may contain the genes for applications in the production of transgenic plants. Further

research is needed in the open field and greenhouse to evaluate the effectiveness of selected strains to control *A. gossypii*.

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Conflicts of Interest

The authors declare that they have no competing interests.

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