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ISOLATION OF ENTOMOPATHOGENIC BACTERIA FROM LARVAE OF A LEPIDOPTERAN SPECIE; GALLERIA MELLONELLA AND STUDY OF THEIR INSECTICIDAL EFFECT

SUMMARY

Few years ago, considerable progress has been made to explain the disappearance of bee colonies, including correct identification of pests involved and the search for more effective and healthy alternatives to protect them. Indeed, our work is based on the isolation, characterization and identification of entomopathogenic bacterial strains of the genus *Bacillus* from larvae of wax moth *Galleria mellonella* reared in the laboratory, with a preliminary study of the use of these entomopathogenic bacteria on the larvae (L5) of *G. mellonella* under controlled conditions. In fact, 9 bacterial strains of the genus *Bacillus* have been isolated. They are spore forming bacteria, Gram, catalase and oxidase positive and present variable responses to the gelatinase test, lecithinase, caseinase, culture in anaerobiosis and growth at different temperatures (45 ° C., 55 ° C., 65 ° C.). From our study, we also find that the strain S4, probably identified as *Bacillus thuringiensis*, has a better effect on the larvae of *Galleria mellonella*. It caused very remarkable symptoms and mortality rates that vary depending on the strain and bacterial concentration tested and the mode of application. Injection of strain S4 for individuals resulted mortality of 83.33%, 75% and 50%, respectively, after treatment with high, medium and low concentration after only 3 days. The comparative examination of the hemolymph test results shows that the injection of the bacteria into the larvae resulted in a significant increase in hemolymph protein and carbohydrate content as compared to controls.

Keywords: *Galleria mellonella*, *Bacillus*, isolation, mortality, haemolymph.

INTRODUCTION

Honeybee pests are known to cause significant losses, and to transmit viral pathogens for which therapies remain nonexistent and continue to be challenging to eradicate (Plettner *et al.*, 2017). The greater wax moth, *Galleria mellonella*

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Linnaeus, is a ubiquitous pest of the honeybee, *Apis mellifera*. The damage caused by *G. mellonella* larvae is severe, and is believed to be one of the contributing factors to the decline in honeybee populations. Previously, the pest was considered a nuisance in honeybee colonies, it is currently widespread, especially in Africa, and the potential of transmitting honeybee viruses has raised legitimate concern (Kwadha *et al.*, 2017). The wax moth was first reported in honeybee colonies of Asian honeybee *Apis cerana* (Paddock, 1918), but later spread to northern Africa, and other parts of the world (Akratanakul, 1987). Shimanuki (1980) and Williams (1997) later described the pests as ubiquitously distributed everywhere beekeeping is practiced.

Use of chemical and fumigant insecticides to destroy this insect are harmful to bee populations (Calderone, 2000). Certain ones pose health risks to the handler and lead to residues in hive products such as honey, rendering the product inconsumable (Ritter and Akratanakul, 2006). More importantly, they are poisonous to honeybee colonies and non-target species (Ritter and Akratanakul, 2006; Charriere and Imdorf, 1999). Previous researchers have explored various biological agents and bio-products including *Bacillus thuringiensis* Berliner (H-serotype V) (Plettner *et al.*, 2017), but evidence for a successful and sustainable biological control agent of *G. mellonella* is still lacking. The objective of the present study is to screen different bacterial strains from the cadavers of the greater wax moth reared on laboratory and testing them against the larva of this insect, in order to explore the toxins of these bacteria.

MATERIAL AND METHODS

Isolation and characterization of bacterial strains

From the larva of the greater wax moth accidentally contaminated in the laboratory during the various manipulations, a few individuals were isolated immediately after their death. These were deposited on the surface of a nutrient agar and then incubated at 32 ° C. 24 hours after incubation, bacterial colonies were obtained around the deposited cadavers. Successive transplants have resulted in pure and well-defined colonies. Only colonies with the macroscopic characters of the genus *Bacillus* are taken into consideration.

Preliminary identification of the isolates took place according to the taxonomic characterization proposed by Guiraud (2003). For this, a macroscopic examination of the colonies constitutes a first step which guides the process of characterization of the bacteria. Microscopic examination consists of microscopic observation in the fresh state followed by simple staining with methylene blue, Gram staining and spore staining (Larpen, 1997, Singleton, 2005). For the biochemical characterization, we used the classical identification tests and the API system. Among the tests performed; Simmons citrate test, Voges Proskauer reaction (VP), methyl red reaction (RM), TSI study, growth at 45 ° C, 55 ° C and 65 ° C (Guiraud, 2003). Among the enzymes researched; catalase, oxidase, caseinase, lecithinase, nitrate reductase (NR) (Geraldine *et al.*, 1981; Graden and Luisetti, 1981; Gerard *et al.*, 2003).

Treatment of larvae of the greater wax moth

Fifth-stage healthy larvae were isolated from the rearing, these were treated by introducing 3 different concentrations of the bacterial suspensions, using 2 modes of application, ingestion and injection. The control and treated larvae are fed with honey, pollen and wax and placed in an oven at 30 ° C. In order to study the efficacy of the two bacterial strains selected on the larvae of the greater wax moth, 3 parameters were chosen; Calculation of the corrected mortality, calculation of LT50 (lethal time for 50% of individuals), study of haemolymphatic composition. To study this last parameter, the treatment of larvae was done by injection of a volume of 20µl of the bacterial suspension by individuals, controls received physiological saline. The haemolymph was taken 4h, 6h and 12h after the treatment. Indeed, a volume of 10 µl is taken at the abdominal end of the insect, 5 µl are used for the determination of proteins and 5 µl for the determination of carbohydrates. The protein assay is carried out according to the method of Bradford (1976). The colorimetric method for the determination of carbohydrate by anthrone described by Bachelier and Gavinelli (1966) was used.

Statistical analysis

The significance of the main effects was determined by analysis of variance (ANOVA). The values ($p \leq 0.05$) are considered statistically significant. A correlation matrix is used to describe the degree of relationship between two variables. The software used is the Statistica.

RESULTS AND DISCUSSION

Characterization of bacterial isolates

The isolation of bacterial strains from *G. mellonella* larvae allowed the selection of 9 bacterial strains of the genus *Bacillus*.

Cultural and macroscopic tests: macroscopic examination on solid media (nutritive agar) showed well-isolated colonies.

Table 1. Cultural characteristics of isolates on solid medium

Isolates	Shape	diameter	Color	Opacity	Elevation	Surface	Odor
1	CIB	punctiform	white	opaque	flat	smooth	Ab
2	CRB	punctiform	cream	opaque	flat	smooth	Ab
3	CIB	1.8mm	cream	opaque	flat	smooth	Ab
4	CIB	1.6mm	white	opaque	flat	smooth	Ab
5	CRB	punctiform	white	opaque	flat	smooth	Ab
6	CRB	1.7mm	white	opaque	flat	smooth	Ab
7	CIB	2mm	white	opaque	Convex	Granular	Ab
8	CIB	punctiforme	cream	opaque	Convex	Granular	Ab
9	CRB	1.3mm	white	opaque	flat	smooth	Ab

*CIB : circular with irregular board, CRB : circular with regular board, Ab : absence

Their appearance is very variable (table 1); they are opaque, cream or white colored colonies with an average diameter of 1.3 to 2 mm. Some strains have punctiform colonies. The elevation differs from one isolate to the other between flat and convex, some colonies have a granular surface although the majority, their surface is smooth, the shape of the colonies obtained is circular with regular board or not.

The study of the cultural characteristics on liquid medium (nutrient broth) after incubation at 32 ° C shows the presence of a cool at the bottom of the tube for most strains with the appearance of a homogeneous disorder and the presence veils and the ring surface (table 2).

Table 2. Cultivation characteristics on liquid medium

Isolates	rings	sails	homogeneous disorder	heterogeneous disorder	cool
S1	+	+	+	-	+
S2	+	+	+	-	+
S3	+	+	+	-	+
S4	+	-	+	-	+
S5	+	+	+	-	+
S6	+	+	+	-	+
S7	+	+	+	-	+
S8	+	-	+	-	-
S9	+	-	+	-	+

(+) : presence

(-) : absence

Microscopic characteristics of the isolates: microscopic observation of the cells after fresh staining, simple staining with methylene blue and Gram staining; has shown that all isolated strains have the shape of a rounded or square rod and are Gram-positive. Malachite green staining indicates that all isolated strains form spores. The spore has an oval shape in a central or terminal position (figure 1).

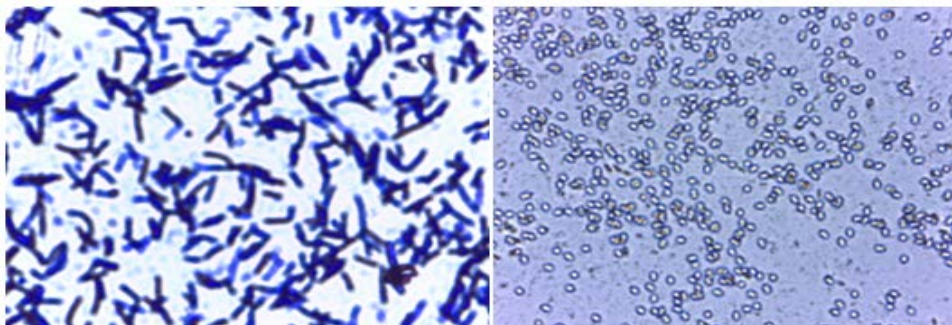


Figure 1: results of observation after Gram staining (right) and staining of spores (left)

Physiological and biochemical characterization: the results of the various biochemical tests carried out (table 3) indicate positive responses by all

the strains for the oxidase, gelatinase, VP, TDA, ONPG, CIT, starch hydrolysis, catalase and caseinase tests , with negative responses for RHA, URA. The responses were variable for NO, MAN, ADH, lecithin, mobility, culture in anaerobiosis and growth at different temperatures (45°C, 55°C, 65°C).

Table 3. Physiological and biochemical characterization of isolated strains

API system	S1	S2	S3	S4	S5	S6	S7	S8	S9
ARA	+	-	+	-	-	-	-	-	+
AMY	+	-	+	-	-	-	-	+	+
MEL	+	-	+	-	-	-	-	-	+
SAC	-	-	-	-	-	-	-	-	+
RHA	-	-	-	-	-	-	-	-	-
SOR	+	+	-	-	+	+	-	-	+
INO	+	-	-	-	-	-	+	+	-
MAN	-	+	+	-	-	-	-	-	+
GLU	-	-	-	-	-	+	-	-	+
GEL	+	+	+	+	+	+	+	+	+
VP	+	+	+	+	+	+	+	+	+
IND	-	-	-	-	-	-	-	-	+
TDA	+	+	+	+	+	+	+	+	+
URE	-	-	-	-	-	-	-	-	-
H2S	-	-	+	+	-	V	V	-	V
CIT	+	+	+	+	+	+	+	+	+
ODC	+	+	+	-	-	+	+	-	-
ADH	+	+	+	-	-	+	+	-	-
ONPG	+	+	+	+	+	+	+	+	+
Other tests									
Gram	+	+	+	+	+	+	+	+	+
Caseine	+	+	+	+	+	+	+	+	+
Lecithine	++	+++	-	+++	++	+	+	-	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+
Mobility	+	+	+	+	+	+	-	+	+
RM	+	+	+	+	+	+	+	+	+
Anaerobic culture	+	+	-	+	-	-	+	-	+
Culture at 45°C	+	v	+	-	v	+	+	-	+
Culture at 55°C	-	-	-	-	-	-	-	-	-
Culture at 65°C	-	-	-	-	-	-	-	-	-

(+): Positive result (-): negative result (v): variable

Several bacteria have been identified as having potential for use in biological control. These entomopathogenic bacteria belong particularly to the Bacillaceae family (Starnes *et al.*, 1993). From our study, 9 bacterial strains of the genus *Bacillus* were isolated from the larvae of *G. mellonella*. After purification, the isolates were identified according to their macroscopic, physiological and biochemical characteristics. Guiraud (2003), Geraldine *et al.* (1981) and Singleton (2005), confirm their affiliation to the genus *Bacillus*. According to Brossard and Terry (1984), *Bacillus* species are ubiquitous microorganisms, the majority grow better at 30 ° C up to 37 ° C, many species

are saprophytes of soil, water, air and plants (Claus and Berkeley, 1986). The strains isolated have all the cultural and microscopic traits of the genus *Bacillus* described by Euzéby (2007). These catalase positive bacteria with variable response to the oxidase test (Guiraud, 2003) are mobile by cilia, their spores are ellipsoidal to cylindrical (Cloutier and Cloutier, 1992). Isolation of bacteria from nymphs *Phyllocnistis citrella* revealed the existence of five different bacterial strains. Their identification showed that the genus *Bacillus* is the most frequently encountered. The pathogenicity test with the bacteria isolated showed high mortality of larvae of *P. citrella* (Saiah *et al.*, 2010).

Evaluation of the effect of bacterial isolates on larvae of *G. Mellonella*

In order to determine the effect of isolated entomopathogenic bacteria on the larvae of *G. mellonella*, two bacterial strains (S3 and S4) were chosen. The individuals treated showed remarkable symptoms (figure 2).



Figure 2. Symptoms observed in individuals treated with bacterial suspensions (A/ appearance deformations in the survivors after the emergence of the butterflies in particular in the wings. B/ blackening and softening of the body just after death due to melanin secretion).

After injection of bacterium *Klebsiella pneumoniae* into the hemolymph of *G. mellonella*, the larvae and hemolymph progressively pigmented during infection indicating the production of melanin by the enzyme phenoloxidase (PO). The activity of this enzyme causes deposition of melanin around bacteria by insect hemocytes, to wrap and isolate it (Insua *et al.*, 2013).

Mortality rate: the use of the two strains S3 and S4 against the L5 larvae of *G. mellonella* gave interesting results. For strain S3, 100% mortality was observed after 9 days for the high dose, whereas for the mean and the low dose, the effects were different depending on the mode of application of the treatment. They are more pronounced in injection-treated individuals, with 90% mortality for the mean dose (compared with 66.66% for ingestion) and 62% for the low dose (compared to 33.33% for ingestion) (figure 3).

Injection of S4 strain to *G. mellonella* caused 83.33%, 75% and 50% mortality respectively after treatment with D1, D2 and D3 after 3 days. In larvae treated with ingestion, a mortality rate of 66 % was observed after treatment with D1, 41% for D2 and 25% for D3, after 9 days. Indeed, *Bacillus thuringiensis*, like *Bacillus popilliae*, *B. alvei*, *B. larvae*, *B. lentimorbus* and *B. sphaericus*,

possess the particular property of inducing mortality in certain insects (Joung & Côté, 2001; Lacoursiere & Boisvert, 2004). These latter justify the harmfulness of the *Bacillus* by the interaction of their toxins with specific receptors on the epithelial cells of the digestive system, which causes the death of the insect following the disruption of the osmotic regulation of these cells.

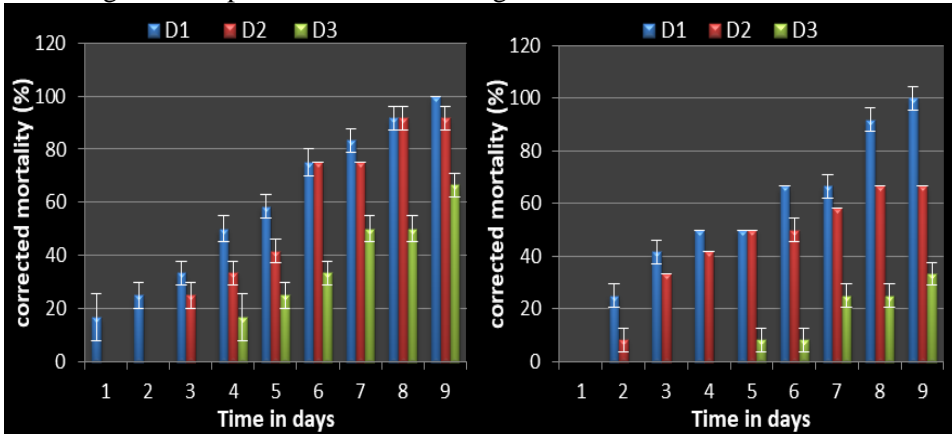


Figure 3: corrected mortality rate in *G. mellonella* larvae treated with strain S3 (left injection, right ingestion) ($D1 = 0.9 \times 10^8$, $D2 = 0.3 \times 10^8$, $D3 = 0.16 \times 10^8$). ANOVA indicate a significant difference in the treated individuals by injection ($F = 0,000017$) and ingestion ($F = 0.000000$) compared to controls. Similarly, the correlation matrix test shows that there is a correlation between the mortality factor and the time factor and also between the mortality factor and the dose factor

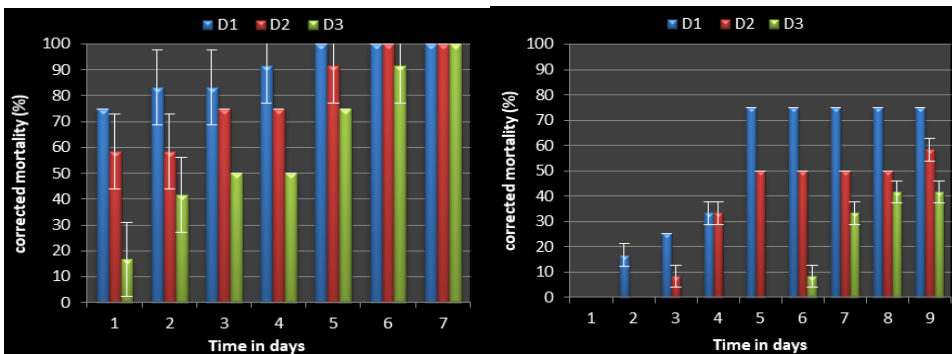


Figure 4: corrected mortality rate in *G. mellonella* larvae treated by strain S4 by injection (left) and ingestion (right). ($D1 = 0.9 \times 10^8$, $D2 = 0.3 \times 10^8$, $D3 = 0.16 \times 10^8$). (A significant difference was demonstrated by the test of variance analysis in the treated individuals by injection ($F = 0.000487$) and ingestion ($F = 0.000000$) compared to controls. As for the correlation matrix test, it indicates a correlation between the mortality factor and the time factor and also between the mortality and dose factor).

The present findings are in agreement with El behery *et al.* (2016) who found that rearing the *G. mellonella* on the different ages of beeswax combs with 4% Neem Azal-T/S showed that all the tested larvae died during the first week of treatment.

LT50 : lethal times for 50% of individuals vary depending on the bacteria, the applied doses and the mode of treatment. The larvae of *G. mellonella* treated with S4 have the lowest TL50s, the TL50s increase with the decrease in doses. TL50s are very short for injection mode compared to ingestion.

Table 4. LT50 values recorded in *G. mellonella* larvae treated with strains S3 and S4

Treatment	Dose	Injection		Ingestion	
Strain		S 3	S4	S3	S4
Doses	D1=0,9*108 ufc/ml	0,76 day	0,44 day	4,14 days	3,98 days
	D2= 0,3*108ufc/ml	2,01 days	0,68 day	4,9 days	7,58 days
	D3= 0,16*108ufc/ml	3,69 days	1,18 day	11,69 days	11,11 days

It has been found after treatment of *G. mellonella* with the polyphenols of Bitter Orange (*Citrus Aurantium*) that this extract showed a valuable efficacy against larvae, an LT50 of 2.34 days was obtained after treatment by dose 20 μ l / ml (Oulebsir-MohandKaci *et al.*, 2016).

Effects of bacteria on the haemolymphatic composition of *G. mellonella* larvae: the hemolymph assay allowed to plot the regression line which allowed to calculate the concentrations of proteins and carbohydrates haemolymphatic in the controls and treated by the strain S4 .

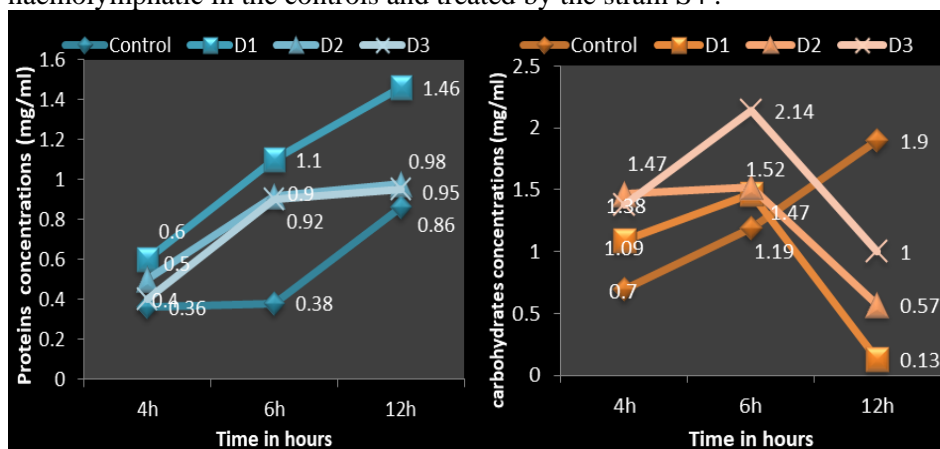


Figure 5: Concentrations (C) of protein and hemolymphatic carbohydrate samples in S4-treated individuals

The injection of the bacterium into the larvae of *G. mellonella*, resulted in a significant increase in the haemolymph protein content compared to the controls, in particular with the high dose. This increase is proportional to the concentration injected. The carbohydrate content also increased in the treats by

the 3 doses compared to the controls and this at 4h and 6h. At 12 h, this concentration was markedly decreased in the treated individuals with a normal evolution in the controls (figure 5).

The results obtained by Oulebsir-Mohand Kaci and Doumandji-Mitiche(2012) show an important decrease of haemolymph protein concentration compared to controls with an increase in carbohydrate concentration, after treatment of larvae of *Locusta migratoria* by *Pseudomonas fluorescens*.

CONCLUSIONS

The isolation of some bacterial strains belonging to the genus *Bacillus* from the dead larvae of *G. mellonella* resulted in the identification of 9 species that differed according to their macroscopic, physiological and biochemical characteristics. These isolates were tested on larvae of the same insect in order to confirm their efficacy and to evaluate their toxicity.

The isolates tested against the L5 larvae of the greater wax moth caused very high mortality rates. Comparative examination of the results of the determination of haemolymph protein and carbohydrate showed a difference between the controls and the treated by the bacteria. Finally, it is clear that isolated bacterial strains represent an interesting step forward in the fight against the greater wax moth because they affect its development and metabolism. It is therefore desirable to study further their ecological and toxicological impacts.

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