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Molluscicidal potential of Bacillus thuringiensis and endogenous bacteria against Theba pisana

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In an agricultural context where the chemical control of pest gastropods is showing its limits, due to the environmental impact of molluscicides and the emergence of resistance, this study explored sustainable biological alternatives. The objective was to identify and characterize the molluscicidal potential of bacterial strains against two major pests in Morocco, *Theba pisana* and *Helix aspersa*. The methodology was based on the isolation of bacterial strains from snail cadavers, followed by laboratory bioassays to evaluate their efficacy in comparison with a commercial strain, *Bacillus thuringiensis* var. *kurstaki* (BT). Virulence was quantified by measuring mortality rates and calculating the median lethal dose (LD50). Potential mechanisms of action were investigated by analyzing the activity of key enzymes (proteases, chitinases, lipases). The results revealed a remarkable efficacy of the BT strain against *Theba pisana*, causing 100% mortality within 21 days, with a calculated LD50 of 1.5×10^{8} CFU/ml. The endogenous strains, identified as *Pseudomonas iridis* (B9) and *Enterobacter aerogenes* (B3), showed more moderate efficacy against the same snail. Regarding *Helix aspersa*, only the BT strain demonstrated lethal activity, although it was limited (34% mortality). The investigation of the mechanisms of action highlighted distinct enzymatic profiles: high protease activity for BT, a pronounced chitinase activity for *E. aerogenes*, and significant protease activity for *P. iridis*. Although promising, these laboratory results do not guarantee success in field conditions, where environmental factors such as temperature, humidity, and UV radiation can affect bacterial viability. It is therefore imperative to validate their efficacy in the field, develop protective formulations, and conduct safety studies before integrating these agents into sustainable control strategies.

Keywords: Biological control, *Theba pisana*, *Helix aspersa*, *Bacillus thuringiensis*, *Pseudomonas iridis*, *Enterobacter aerogenes*, Enzymatic activity

INTRODUCTION

Molluscs represent the second largest phylum in the animal kingdom, playing a significant ecological role. They are characterized by a soft body, often protected by a calcium-rich shell (Zala et al., 2018). Among the diverse classes within this phylum, Gastropoda, which includes snails and slugs, is the most prominent, accounting for 80% of all mollusc species (Srivastava, 1992).

In agricultural settings, however, terrestrial gastropods are recognized as notorious pests due to their significant impact on a wide variety of crops (Das et al., 2020). Their feeding activity causes substantial damage to vegetables, field crops, ornamental plants, and fruit trees (Godan, 1983; Carlsson et al., 2004), leading to major economic losses. This damage is not limited to direct consumption of plant tissues but also includes the contamination of harvests with their bodies, mucus, and excrement, thereby reducing the quality and marketability of agricultural products

(South, 1992).

Traditionally, the management of these pests has relied heavily on chemical molluscicides. However, this approach faces critical limitations, including negative environmental impacts and the emergence of resistance in pest populations. This context has spurred a growing interest in biological control, a strategy that employs natural enemies to reduce pest populations and their impact (Bale et al., 2008). Among the various biocontrol agents, pathogenic microorganisms, particularly bacteria, have shown considerable potential.

Several bacterial genera, such as Bacillus, Pseudomonas, and Serratia, have demonstrated molluscicidal activity. These bacteria can kill gastropods through various mechanisms, including the production of toxins, tissue-degrading enzymes, or systemic infections (Wilson and Grewal, 2005). Notably, certain strains of Bacillus thuringiensis (Bt) are known to produce protein toxins that are lethal to specific snail species, highlighting their potential for targeted and safe biocontrol (Gingichashvili et al., 2017).

This study aims to explore this potential by identifying and characterizing the molluscicidal activity of bacterial strains against two major agricultural pests in Morocco: Theba pisana and Helix aspersa. We evaluated the efficacy of a commercial strain, Bacillus thuringiensis var. kurstaki (BT), alongside two endogenous strains isolated from snail cadavers, Pseudomonas iridis (B9) and Enterobacter aerogenes (B3). The investigation also sought to elucidate the potential mechanisms of action by analyzing key enzymatic activities (protease, chitinase, and lipase) of the selected bacteria. The findings are intended to contribute to the development of sustainable and effective strategies for the management of pest snails in agriculture.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The commercial strain Bacillus thuringiensis var. kurstaki (BT) was used as a reference treatment. Nine endogenous bacterial strains were isolated from the cadavers of Theba pisana snails collected from the pedagogical farm of the National School of Agriculture (ENA) in Meknes, Morocco.

For isolation, tissues from snail cadavers were suspended in sterile phosphate-buffered saline (PBS) and agitated for 60 minutes. The supernatant was then plated on Luria-Bertani (LB) agar, composed of tryptone (10 g/L), yeast extract (5 g/L), NaCl (10 g/L), and agar (15 g/L). Plates were incubated at 28°C for 24 to 48 hours. Colonies with distinct morphologies were selected and purified through successive subculturing. Purified isolates were characterized morphologically and by Gram staining, observed under an Euromex bScope series microscope. Pure cultures were maintained on LB agar slants at 4°C for future use.

Snail Collection and Acclimatization

Adult specimens of Theba pisana and Helix aspersa were collected manually from a ‘Newhall’ variety citrus orchard at the Moulay Abdelaziz domain in the Gharb region, Morocco. Snails were gathered from specific micro-habitats, including shaded parts of trees, areas near drip irrigation lines, and weed cover.

The collected snails were transported to the laboratory in a 5-liter plastic box and transferred to larger containers with a sterile, moist, sandy-loam substrate for acclimatization. They were acclimatized for 14 days under controlled conditions (24°C, 70% relative humidity, 12:12 h light:dark photoperiod). During this period, they were fed fresh lettuce (Lactuca sativa). To eliminate potential pesticide residues, the lettuce leaves were systematically washed and immersed in distilled water for 24 hours before being distributed.

Pathogenicity Bioassays

A preliminary screening was conducted on T. pisana to identify the most virulent bacterial isolates. A total of 396 snails were distributed into 66 experimental units (6 snails per unit). The experiment followed a completely randomized design (CRD) with three replicates for each of the 11 treatments (9 endogenous isolates, 1 BT strain, and 1 control).

Experimental units were prepared aseptically under a laminar flow hood. Lettuce leaves were surface-sterilized by immersion in a 3% sodium hypochlorite (NaOCl) solution for five minutes, followed by three successive rinses in sterile distilled water. Each sterilized leaf was placed in a sterile plastic box with fine perforations to allow for gas exchange.

Bacterial suspensions were prepared from 24-hour cultures and standardized to a concentration of approximately 10^8 CFU/mL by adjusting the optical density (OD) to 2.0 at 600 nm using a PG Instruments T60 UV-Visible Spectrophotometer. Two application methods were evaluated:

• Residual Film Technique: 1 mL of the bacterial suspension was sprayed onto the internal surfaces of the experimental box. Snails were provided with a fresh, untreated lettuce leaf, which was replaced every two days.

• Leaf Spray Technique: 1 mL of the bacterial suspension was sprayed uniformly onto the lettuce leaf, which was then offered to the snails until fully consumed, after which it was replaced with fresh, untreated lettuce.

Control groups were treated with sterile distilled water. Snail mortality was recorded weekly for 28 days. The corrected mortality percentage was calculated using Abbott's formula (Abbott, 1925).

Determination of Median Lethal Dose (LD_{50})

A dose-response bioassay was performed on T. pisana using the most virulent strains (BT, B9, and B3). Five concentrations were tested: 10^6, 10^7, 10^8, 10^9, and 10^{10} CFU/mL, following the leaf spray protocol described above.

Molecular Identification of Bacterial Isolates

The most effective isolates (B3 and B9) were identified by 16S rRNA gene sequencing. Genomic DNA was extracted following the protocol described by Yaish et al. (2015). A partial fragment of the 16S rRNA gene was amplified using universal primers 27f and 1492r. The 25 µL PCR reaction mixture contained 12.5 µL of 2X Master Mix, 1.25 µL of each primer (10 µM), 2.5 µL of template DNA, and 7.5 µL of sterile water. PCR products were visualized by electrophoresis, and positive amplicons were sequenced using a SeqStudio capillary analyzer (Applied Biosystems).

Screening for Enzymatic Activity

Selected strains were screened for their ability to produce key hydrolytic enzymes. The diameters of halos and colonies were measured with a Kendo digital caliper to calculate the relative enzymatic index.

• Protease Activity: Assessed on skim milk agar according to Kumar et al. (2005). A clear zone around colonies after 72 hours at 28°C indicated proteolytic activity.

• Chitinase Activity: Tested on colloidal chitin agar as described by Saima et al. (2013). A transparent halo around colonies after 5 days at 28°C indicated chitinolytic activity.

• Lipase Activity: Evaluated on LB agar with 1% Tween 20, following Mina et al. (2020). An

opalescent zone around colonies after 72 hours at 28°C indicated lipase production.

Statistical Analysis

Data were analyzed using SPSS software (version 25). The effects of bacterial strain, dose, and application method on snail mortality, as well as enzymatic activities, were analyzed using Analysis of Variance (ANOVA). Means were compared using the Student-Newman-Keuls (SNK) post-hoc test ($p < 0.05$). LD_{50} values were estimated using Probit analysis.

RESULTS

Efficacy of Bacterial Strains against Theba pisana

Preliminary Screening of Isolates

In the preliminary screening, only three of the ten tested bacterial strains showed lethal effects against T. pisana: BT, B9, and B3. The commercial strain BT was the most virulent, causing 100% corrected mortality by day 21. The endogenous strains B9 and B3 exhibited moderate to low efficacy, reaching a maximum mortality of 33.3% and 16.7%, respectively, by day 28 (Figure 1). The remaining isolates (B1, B2, B4, B5, B6, B7, B8) showed no molluscicidal activity, with results indistinguishable from the control Statistical analysis (ANOVA) revealed that the bacterial strain was the only factor with a highly significant effect on mortality. The application method (residual film vs. leaf spray) and the interaction between strain and method were not statistically significant ($p = 0.777$ and $p = 1.000$, respectively). The post-hoc SNK test confirmed that BT was significantly more effective than all other strains. Strains B9 and B3 formed a group with low to moderate efficacy, while the other strains were statistically similar to the untreated control. Snails killed by the effective strains are shown in Figure 2.

Dose-Response Relationship and LD_{50} Determination

The three effective strains (BT, B9, B3) were tested at five different concentrations against T. pisana. A clear dose-response relationship was observed for all three strains, with mortality increasing with concentration (Figure 3). The BT strain showed a dramatic increase in efficacy, reaching approximately 73% mortality at concentrations of 10^8 CFU/mL and higher. Strain B9 showed a more modest, gradual increase in mortality, while strain B3 was the least effective, causing less than 10% mortality even at the highest concentration.

Probit analysis was used to estimate the median lethal dose (LD_{50}). For the BT strain, the LD_{50} was calculated to be 1.49×10^8 CFU/mL (Table 1). For strains B9 and B3, mortality never reached the 50% threshold, even at the highest concentration tested (10^{10} CFU/mL). Therefore, their LD_{50} values are considered to be greater than 10^{10} CFU/mL, confirming their lower pathogenicity against T. pisana.

Efficacy of Bacterial Strains against Helix aspersa

When tested against H. aspersa, only the BT strain demonstrated lethal activity. Its efficacy was dose-dependent, with mean corrected mortality increasing from 17% at 10^8 CFU/mL to 27% at 10^9 CFU/mL (Figure 4). In contrast, strain B9 showed no pathogenic effect on H. aspersa, with zero mortality observed at both concentrations tested. The ANOVA confirmed that the strain was the only significant factor influencing mortality.

Molecular Identification of Selected Strains

The two most effective endogenous isolates, B3 and B9, were identified through 16S rRNA gene

sequencing. Isolate B3 was identified as Enterobacter aerogenes, and isolate B9 was identified as Pseudomonas iridis (Table 2).

Enzymatic Activity of Selected Strains

Protease Activity

All three selected strains (BT, B9, B3) exhibited protease activity, but at significantly different levels. The BT strain displayed the highest proteolytic activity (mean relative index = 0.33), followed by P. iridis B9 (0.23), and lastly E. aerogenes B3 (0.14) (Figure 6). The SNK test confirmed that the differences between all three strains were statistically significant.

Chitinase Activity

For chitinase activity, a different hierarchy was observed. E. aerogenes B3 showed the highest chitinolytic activity (mean index = 0.38), followed by BT (0.32), and P. iridis B9 (0.25) (Figure 7). The SNK test revealed that the activity levels of all three strains were significantly different from one another.

Lipase Activity

All three strains demonstrated similar levels of lipase activity. The mean relative indices for E. aerogenes B3 (0.31), P. iridis B9 (0.29), and BT (0.30) were very close (Figure 8). Statistical analysis confirmed this observation, showing no significant difference in lipase production among the three strains ($p = 0.882$).

DISCUSSION

The use of microorganisms, such as bacteria and fungi, has proven to be an effective control method against terrestrial snails. The application of these microbial agents offers an alternative that avoids the harmful use of chemical molluscicides, making them viable options for integrated pest management programs (Abo-Elwfa et al., 2024).

The foundational results of this study show 100% mortality in Theba pisana on the 21st day of treatment with a strain of Bacillus thuringiensis kurstaki, with a calculated median lethal dose (LD_{50}) of 1.49×10^{8} CFU/mL. These findings have a direct and significant parallel in the work of Hendi et al. (2015), who determined a median lethal concentration (LC_{50}) of 7.3×10^{8} viable spores/mL after 13 days of treatment on a native strain of B. thuringiensis isolated from the cadavers of T. pisana. The comparison of these two values, although expressed in slightly different units, is illuminating: in both cases, the order of magnitude is 10^{8}, which is a strong indicator of a fundamental and predictable susceptibility of T. pisana to B. thuringiensis infection. The mortality kinetics reinforce this interpretation: achieving 100% mortality in 21 days in our trials perfectly aligns with the observations of Hendi et al. (2015), who reported total mortality in 20 days. This temporal consistency suggests a characteristic and reproducible infection dynamic.

However, the relevance of a biological control agent can only be fully established if its efficacy in the laboratory translates into tangible control under agricultural conditions. Field data confirm this potential. A study by Mostafa et al. (2023) evaluated the efficacy of the commercial product «Protecto» whose active ingredient is B. thuringiensis var. kurstaki, the same subspecies used in our trials. The application of this product on orange trees infested with T. pisana resulted in a population reduction of up to 81.3% by spraying and 70.2% with poison baits. This correlation between high virulence in the laboratory and success in real-world conditions establishes a complete validation chain.

In contrast to the clear results on T. pisana, the evaluation of the efficacy of B. thuringiensis against Helix aspersa reveals a complex and contradictory scientific picture. Our data show moderate and dose-dependent mortality, increasing from 16% to 27%. These observations are consistent with those of Rabeih et al. (2005), who reported a mortality rate of 47.8%. On the other hand, a series of rigorous studies by Kramarz et al. (2007) reached a radically different conclusion, showing a total absence of effect of the purified Cry1Ab toxin on H. aspersa. The most plausible reconciliation for these results lies in the distinction between the use of a «commercial formulation» and that of a «purified toxin». Complete formulations contain, in addition to Cry proteins, viable spores and a variety of metabolites (exotoxins, chitinases, phospholipases) that could act synergistically to cause the observed mortality.

A cross-cutting analysis highlights marked differences in susceptibility among gastropod genera. The genus Monacha appears exceptionally vulnerable, with several studies reporting remarkably low LC_{50} values in the range of 10^6 cells/mL (Abo-Elwfa et al., 2024; Gaber et al., 2022; Genena and Mostafa, 2008). These values are systematically one to two orders of magnitude lower than those obtained for T. pisana (order of 10^8). This gap is likely related to fundamental biological differences, such as intestinal physiology or the presence of specific receptors for Cry toxins.

The endogenous strains Pseudomonas iridis (B9) and Enterobacter aerogenes (B3) showed more moderate efficacy against T. pisana, with LD_{50} values greater than 10^{10} CFU/mL. Analysis of their potential mechanisms of action through their enzymatic profiles is instructive. For E. aerogenes (B3), chitinolytic activity was the highest, suggesting that chitinolysis is its primary attack strategy. Given that the genus Enterobacter is a natural component of the snail gut microbiome (Charrier et al., 2006), the B3 strain could act as an opportunistic pathobiont. Its high chitinase production would allow it to digest chitin-rich barriers, such as the radula, to cross the epithelium and cause systemic infection (Hegedus et al., 2009). In contrast, P. iridis (B9) bases its virulence on significantly higher protease production. The genus Pseudomonas is known to secrete a variety of proteases that cause significant tissue damage (Doring et al., 2001). In T. pisana, these proteases could have two critical targets: the mucus, a glycoprotein-rich barrier (Arias et al., 2025), and the digestive gland, a vital organ whose damage by proteases can lead to a lethal metabolic collapse (Abd-ElAzeem et al., 2023).

CONCLUSION

This study identified and characterized the molluscicidal potential of several bacterial strains against the major snail pests Theba pisana and Helix aspersa, providing a basis for developing sustainable biological control alternatives to chemical molluscicides. Our laboratory bioassays revealed that Bacillus thuringiensis var. kurstaki (BT) is a highly virulent agent against T. pisana, causing 100% mortality with a calculated LD_{50} of 1.49×10^8 CFU/mL. In contrast, its lethal activity against H. aspersa was moderate and limited. The endogenous strains, identified as Pseudomonas iridis (B9) and Enterobacter aerogenes (B3), showed more modest efficacy against T. pisana.

The investigation into the mechanisms of action highlighted distinct enzymatic profiles, suggesting complementary biocontrol strategies. BT exhibited high protease activity, while E. aerogenes was distinguished by its strong chitinase production, and P. iridis showed significant protease activity. These findings point to different pathways of pathogenesis, from the degradation of protective tissues to the disruption of essential structures like the intestinal membrane.

However, these promising laboratory results must be interpreted with caution, as environmental factors such as temperature, humidity, and UV radiation can significantly affect bacterial viability and virulence in the field. Therefore, future research should focus on validating the efficacy of these strains under field conditions, developing protective formulations to enhance their persistence, and conducting comprehensive ecotoxicological studies to ensure their safety for non-target organisms. Such steps are imperative before these bacterial agents can be successfully

integrated into sustainable Integrated Pest Management (IPM) programs for the control of pest snails.

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