

Use of Nanotechnology Associated With Natural Products as a Strategy for the Control of *Plutella Xylostella* (Linnaeus, 1758) (Lepidoptera, Plutellidae)

Alini de Almeida

dealmeidaalini@gmail.com

UFPR: Universidade Federal do Parana <https://orcid.org/0000-0002-9147-4847>

Edinéia Paula Sartori Schmitz

UFFS: Universidade Federal da Fronteira Sul

Gisele Louro Peres

UFFS: Universidade Federal da Fronteira Sul

Adelita Maria Llinzmeier

UFFS: Universidade Federal da Fronteira Sul

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Abstract

The diamondback moth is a cosmopolitan microlepidoptera considered one of the world's most important pests of Brassicaceae. In Brazil, losses caused by this species reach one billion dollars per year. Despite the existence of natural control methods such as plant-based products and entomopathogenic fungi and bacteria, the synthetic pesticides are still the main products used to its control, which are associated to numerous harms to health and the environment. In this scenario, nanotechnology combined with natural products are promising for the formulation of new natural insecticides so, the main aim of this study was to test a new a natural nanotechnological insecticide based on *Lithothamnion calcareum* (Corallinaceae), a red alga associated with guar-guar, a natural biopolymer, to combat or control individuals of *Plutella xylostella* (Linnaeus 1758) the so-called seaweed-biopolymer solution (SbS). This solution and its respective controls at different concentrations were subjected to insecticidal viability tests by contact and ingestion on second instar larvae of *P. xylostella*. In the contact test, after 13 hours of exposure, 100% of the individuals were dead. In the ingestion test there was a decrease in pupal biomass and the number of eggs deposited. Therefore, the solutions at concentrations of 2.5% and 1.25% proved to be efficient in controlling *P. xylostella*, indicating that the seaweed-biopolymer solution can be employed as a new strategy for control and minimization of the damage caused by this pest.

Introduction

Plutella xylostella (Linnaeus 1758) (Lepidoptera, Plutellidae), popularly known as diamondback moth, is a cosmopolitan insect, considered one of the most important Brassicaceae pests in the world (Boo et al. 2018). The short life cycle, of approximately 28 days, combined with high reproductive rate allowing the occurrence of several generations throughout the year, makes this species damage up to 100% to host plants reaching five billion of dollars in losses per year (Takelar e Shelton 1993; Castelo-Branco 2001; Ulmer et al. 2002; Torres et al. 2006; Zhou et al. 2011). These losses are caused by the larval stage, which damages the hostplant leaves causing devaluation and unfeasibility to the commercialization.

Even with the availability of biological methods, synthetic pesticides are still the main resource used to control of *P. xylostella*. However, studies indicate that the inappropriate use of these products generates negative impacts on the environment and human health (Dutra et al. 2017), promotes the pest resistance and cause the elimination of their natural enemies (Pinheiro et al. 2020). In this sense, the use of new technologies associated with natural products contributes to discovering and formulating new products. In this scenario, nanotechnology emerges as an innovative potential for several areas, including agribusiness. Studies using this technology have increasingly shown that nanotechnology is viable for the control or minimization of damage caused by pests and diseases that compete with cultivars (Coelho 2017), such as in the formulation of new extracts and essential oils of plants, development of controlled release compounds, nematode control, among others (Lopes et al. 2017).

Among the natural products are the plants compounds as the most widely researched, the biopolymers or natural polymers and seaweed flour (Soltani et al. 2021). Biopolymers are macromolecules made up small and simple repeated structural units called monomers. Guar gum is an example of a natural polymer, non-ionic and hydrophilic galactomannan polysaccharide obtained from the endosperm of *Cyamopsis tetragonolobus* (L.) Taub (Leguminosae, Papilionoideae) seeds. Furthermore, is considered low-cost, renewable, non-toxic and biodegradable element, and has great economic and environmental importance (Dai et al. 2020). It is composed mainly of galactomannans (75–80%), water (12%), and protein (5–6%), it has a long polymer chain, high molecular weight, and is widely available in nature (Thombare et al. 2016).

The seaweed *Lithothamnium calcareum* (Pallas) Areschoug, 1852 (Florideophyceae: Corallinales, Corallinaceae), is typically reddish and crystallizes calcium carbonate in its cell walls (Cabioch 1969). It has high content of calcium, sulfur, and potassium in its composition, in addition to having more than 20 elements in variable amounts such as zinc, iron and magnesium (Costa Neto et al. 2010). Furthermore, the exopolysaccharides, hydrophobic biomolecules and hormones contained in the composition of algae have antibacterial, anti-fungal, antiviral, anti-protozoan, nematicides properties (Bhattacharjee 2023).

Given the properties and characteristics of the natural products mentioned above, together with the scarcity of research related, mainly, to the use of *L. calcareum* and the great worldwide demand for the application of pesticides to control insect pest populations, which culminate in serious environmental problems, it is necessary to take a new look at control alternatives that are less harmful to the environment and human health. Therefore, studies on the use of new technologies such as nanotechnology associated with natural products such as *L. calcareum* and biopolymers such as guar gum (*Cyamopsis tetragonolobus* L.), become a promising alternative for the management of populations of *P. xylostella*. Thus, in this study, based on tests carried out in the laboratory, we sought to verify whether the biopolymeric solution of *L. calcareum* (Seaweed-biopolymer Solution) has insecticidal potential on *P. xylostella*.

Material and Methods

Insect rearing

The creation started from larvae and pupae collected in an organic kale plantation in the municipality of Realeza, Paraná, Brazil. The stock rearing was kept at a temperature of 25 ± 2 °C, relative humidity of $60 \pm 2\%$, and photoperiod 12/12 in the laboratory 101 of the Universidade Federal da Fronteira Sul, campus Realeza, Paraná. The procedure for rearing of *P. xylostella* follows the methodology proposed by Barros et al. (2012).

Production of the Seaweed-biopolymer Solution

The production of the seaweed-biopolymer solution (SbS) consisted of three steps: the first refers to the production of the *L. calcareum* solution, the second consists of the production of the biopolymer solution, and finally, the third step corresponds to the interaction of the biopolymer solution with the *L. calcareum* solution, thus obtaining the SbS. For the production of the *L. calcareum* solution (Step 1), 5g of seaweed flour were diluted in 250 ml of acetic acid (P.A). The solution was heated to 40°C, under constant agitation for 24 hours to the complete extraction of the compounds, filtered and reserved. Then (Step 2), 0.1g of guar gum (biopolymer) were added to a volumetric flask, which was calibrated with 100 ml of distilled water and kept under constant agitation for 24 hours at room temperature for the complete solubilization of the biopolymer. After, to miniaturize the particles in solution to the nanometric scale, the biopolymer solution was subjected to ultrasound treatment in an ultrasonic processor model MARCONI, UP 200S with a large gauge tip, for 3 minutes and amplitude of 100%, since results obtained in previous tests indicate this as the best time for miniaturization of guar gum. Finally (Step 3), the complexation of the solution of *L. calcareum* and guar gum was carried out, in which a solution at concentration of 10% was prepared, which consisted of the complexation of 1 ml of the solution of *L. calcareum* to 9 ml of the solution of nanoparticles of biopolymer forming the SbS. From this SbS, new solutions at concentrations of 1.25%, 2.5%, and 5.0% were prepared which were subjected to agitation for approximately 4 hours and then used in the tests with *P. xylostella*. The chemical description and analysis of the SbS can be found in Almeida et al. (in preparation).

In each test (see below) the SbS at the mentioned concentrations and their respective controls (also in the same concentrations 1.25%, 2.5%, and 5.0%), were used, being them, i) solution only of biopolymer, ii) solution only of flour seaweed, iii) only acetic acid solution and, iv) distilled water as a negative control, totaling 13 treatments.

Contact Test

For the contact test, 10 larvae of second instar of *P. xylostella* were placed in Petri dishes, along with previously sanitized kale leaf discs (8 cm² Ø). Sanitation was performed in a 5% sodium hypochlorite solution for 5 minutes, then the leaves were rinsed in running water and dried with filter paper. Subsequently, with the aid of a 100 ml spray bottle, approximately 0.324 ml of the SbS as well as the controls were sprayed onto the larvae in each plate, at a 30 cm distance. The larvae were evaluated under a stereoscopic microscope every hour after the contact with the solution until 16 hours of exposure to verify whether there was mortality. Mechanical stimuli were performed on the larvae with a brush, which was only considered dead when no movement was observed.

Ingestion Test

For the ingestion test, kale leaf discs (8 cm² Ø) previously sanitized as described above, were immersed in the SbS as well as in their respective controls, for 60 seconds and placed on filter paper to remove excess solution.

Then, 10 larvae of second instar of *P. xylostella* were kept in Petri dishes and fed daily with kale leaf discs, also treated daily with SbS, as described above, until pupae were obtained. After 24 hours of obtaining the pupae, 35 pupae from each treatment were weighed using a Shimadzu AUY2020 precision electronic scale with five decimal places. All pupae obtained were individually transferred to test tubes until the emergence of adults, which were then sexed through terminalia differentiation (Capinera 2008). Then, six couples were formed with adults of up to 24 hours of emergency for each treatment. The couples were transferred to laying cages containing cotton soaked in a 10 mg/ml honey solution, a filter paper disc, and one cabbage disc (8 cm² Ø). The cages were monitored daily, and the laying discs were replaced daily for four days (Ferreira et al. 2021) to verify the fecundity of the females by counting the number of eggs. The couples were kept in cages and fed, following the rearing methodology, being followed up until their death to verify their longevity. In addition, the following parameters were evaluated: larval viability, which was determined by the percentage of larvae that reached the pupal stage; pupal viability, corresponding to the percentage of adults that emerged from the pupae and, pupal weight (Silva et al. 2019).

In each of the tests (contact and ingestion), for each of the 13 treatments, three repetitions were performed, each repetition being represented by three Petri dishes containing 10 larvae in each, totaling 30 larvae per repetition and a final N of 1,170 larvae of *P. xylostella* used in each experiment.

Data Analysis

The results obtained from the tests were submitted to statistical analysis performed in the Past4Project® statistical program. Data normality was assessed using the Shapiro-Wilk test. Confirming the non-normality of the data, the non-parametric Kruskal-Wallis test was performed, followed by the Mann-Whitney test.

Results

Contact Test

Evaluating the efficiency of the SbS, it was verified that in the contact test, all solutions caused 100% mortality of *P. xylostella* larvae within 13 hours after exposure. However, only the solutions at concentrations of 1.25% and 2.5% differed significantly ($p < 0.05$) from the control treatments (Table I). There was no significant difference ($p = 0.9431$) when comparing the efficiency between the SbS at 5.0%, 2.5%, and 1.25% (without considering the controls). However, it was observed that the SbS at 5.0% and their respective controls caused injuries to the kale leaves (burned aspect)

after contact with the solution, possibly due to the higher concentration of acetic acid. In this way, to the Ingestion Test (see below) will be shown the results only to SbS at 1.25% and 2.5% concentrations.

Table I. Evaluation of mortality of *P. xylostella* larvae by contact with Seaweed-biopolymer Solution (SbS) at different concentrations and their respective controls.

Treatments	1.25%			2.5%		
	M _e	M _d	p	M _e	M _d	P
SbS	5.625	6	-	5.625	6	-
CFA	0.625	0	0.00054	0.5625	0	0.00038
CBP	0.0625	0	0.00002	0.25	0	0.00011
CAA	0.5	0	0.00029	1.5625	1	0.01072
H₂O D	0.0625	0	0.00002	0.0625	0	0.00002

p values in bold indicate significant difference ($p < 0.05$) in the same column about the SbS. CFA (seaweed flour control), CBP (biopolymer control) and CAA (acetic acid control), H₂O D (distilled water). M_e (mean mortality per hour), M_d (median).

Ingestion Test

When the larvae were fed with kale leaves treated with the SbS at different concentrations, mortality was lower than that observed in the contact test. There was no significant difference in larval viability for SbS and their respective controls at concentrations of 2.5% and 1.25% (Solution 2.5% $p = 0.1966$; Solution 1.25% $p = 0.1658$). Also, there was no significant difference on the pupal viability at different SbS concentrations and their controls (2.5% Solution $p = 0.5592$; 1.25% Solution $p = 0.6504$).

Although parameters such as larval and pupal viability were not significantly altered when the larvae fed on leaves treated with the SbS, significant changes were observed regarding the average pupal weight (Solution 2.5% $p = 7.19^{-9}$ and 1.25% Solution $p = 1.65^{-12}$) and female fecundity (2.5% Solution $p = 4.81^{-6}$ and 1.25% Solution $p = 4.66^{-10}$) at both concentrations. About the average pupae weight, there was a significant increase in weight for both solutions in relation to the negative control and only the SbS at 1.25% differed significantly from all the control treatments, with a weight reduction compared to those observed in the control treatments (except negative control) (Table II).

Table II. Effect of ingestion of kale leaves treated with a Seaweed-biopolymer Solution (SbS) at different concentrations and their respective controls on pupae weight, fecundity, and longevity of *P. xylostella*.

Treatments	Average weight ^a (mg)			Fecundity			Longevity		
	Pupae			Laid eggs ^b			Adults ^c		
	Mean	Median	p	Mean	Median	p	Mean	Median	p
SbS 2.5%	0.00022 ^a	0.00023	-	7.76 ^a	2.5	-	12.5 ^a	1	-
CFA 2.5%	0.00021 ^a	0.00021	0.70660	17.06 ^b	16.64	0.00374	11.8649 ^a	2	0.56870
CAA 2.5%	0.00031 ^b	0.00031	0.73270	18.87 ^{bc}	15	0.00507	11.6087 ^a	2	0.26280
CBP 2.5%	0.00021 ^a	0.00022	0.00008	17.1 ^b	12	0.00018	10.1064 ^a	2	0.24490
H₂O D	0.00018 ^c	0.00019	0.02606	23.76 ^c	24	0.00000	9.36735 ^a	3	0.13830
SbS 1.25%	0.00023 ^a	0.00023	-	7.36 ^a	7	-	8.84375 ^a	2	-
CFA 1.25%	0.00026 ^b	0.00027	0.04201	9.933 ^a	5.5	0.95860	10.7619 ^a	3	0.88330
CAA 1.25%	0.00027 ^b	0.00027	0.00022	22.63 ^{bc}	20	0.00016	10.878 ^a	3	0.09594
CBP 1.25%	0.00028 ^b	0.00029	0.00640	17.2 ^b	14	0.00000	9.93548 ^a	1	0.75320
H₂O D	0.00018 ^c	0.00019	0.00024	23.7 ^{bc}	24	0.00000	9.36735 ^a	3	0.80310

p values in bold indicate significant difference ($p < 0.05$) in the same column about the seaweed-biopolymer solution and their respective controls. SbS: Seaweed-biopolymer Solution; CFA: Seaweed Flour Control; CAA: Acetic Acid Control; CBP: Biopolymer Control; H₂O D (negative control, distilled water). Means followed by different letters in each column indicate differences ($p < 0.05$) between treatments according to the Mann-Whitney test.

Regarding the fecundity, there was a significant reduction in the number of eggs laid by females treated in both concentrations of the SbS compared to the negative control, with a reduction of about 1/3 in the number of eggs laid (Table II). Concerning the adult's longevity, no significant difference was observed between the SbS concentrations (Solution 2.5% $p = 0.2493$ and Solution 1.25% $p = 0.3581$) (Table II).

Discussion

Seaweed extracts are used in agriculture as biostimulants and fertilizers, where plants become more resistant to diseases caused by insects and microorganisms after application (Espinosa-Antón et al. 2020). However, the use of macroalgae in agriculture is still limited due to its low exploitation in the formulation and commercialization of natural agricultural products (Carvalho e Castro 2014). As far as we know, this is the first work that tested the insecticidal effect of *L. calcareum* combined with nanotechnology to control a pest.

According to the analyzes carried out in the present study, the SbS was efficient and could be used as a method to controlling *P. xylostella* populations. Studies carried out by Thakur et al. (2004), point out that extracts obtained from marine organisms have lethal effects on the larvae of some arthropods. Some works indicate that extracts of the seaweeds *Acrosiphonia orientalis* (J.Agardh) P.C.Silva, 1996, *Padina tetrastratica* Hauck, 1887 and *Centroceras clavulatum* (C.Agardh) Montagne, 1846, significantly reduced the number of larvae of *Culex quinquefasciatus* Say, 1823 (Diptera, Culicidae) (Manilal et al. 2009). The same was observed by Ravikumar et al. (2011), who determined that ethanolic extracts of *Enteromorpha intestinalis* (Linnaeus) Nees, 1820, *Dictyota dichotoma* (Hudson) J.V. Lamouroux,

1809 and *Acanthopora spicifera* (M. Vahl) Borgesen, 1910, demonstrated larvicidal activity against *Aedes aegypti* (Linneus 1762) (Diptera, Culicidae). When using the extract of *Caulerpa racemosa* (Forsskål) J. Agardh, 1873, larvicidal activity was observed on *Anopheles stephensi* Liston, 1901, *Aedes aegypti* and *Culex quinquefasciatus* (Ali et al. 2013).

The results mentioned above agree with the data obtained in the present study, in which the SbS caused 100% mortality of the larval stage of *P. xylostella* when it was sprayed on individuals. It is important to mention that at the concentration of 5.0%, the mortality is probably due to the higher concentration of acetic acid present in the solution since it did not differ significantly from the controls. It is also worth mentioning that qualitatively, the SbS at 5.0% caused injuries to kale leaves used for both contact and ingestion tests, which was not observed at concentrations of 2.5% and 1.25%. Thus, the use of the 5.0% solution is not indicated.

The main chemical component in the structure of the external cuticle of arthropods is chitin (Farnesi et al. 2012), a natural polysaccharide characterized by positive electrical charges (Dutta et al. 2004; Park e Kim 2010; Croisier e Jérôme 2013). This condition may have favored the interaction of the macromolecules of the SbS with the external cuticle of the larvae due to the attraction between the positive and negative poles, which may explain the high mortality rate of the larvae observed in the contact test. The same may have occurred in the ingestion test since the peritrophic membrane that covers and protects the mesentery of arthropods has an alkaline pH and is mostly composed of chitin (Pinto 2019). According to Maroneze and Gallegos (2009), the pupal weight is directly related to the quality and quantity of food that is consumed by the insect during the larval period, in this way it is believed that the negative charges of the SbS, together with the Acid pH when ingested by larvae may have favored the hydrolysis of chitin and corrupted the peritrophic membrane, which affected the absorption of nutrients, so biological parameters such as decreased oviposition and average pupae weight have been altered. In addition, it is known that guar gum has a high-water retention capacity (Vernaza e Chang 2013), which may explain the higher weight of pupae fed with leaves treated with the SbS when compared to the negative control.

Although the use of natural products may be less efficient than synthetic insecticides in promoting acute effects in certain periods of insect life, their use has advantages such as low persistence in the environment and lower toxicity to non-target organisms (Pontual et al. 2012; Camaroti et al. 2018). In addition to affecting larval viability, alteration in pupal weight, and decrease in *P. xylostella* oviposition, the SbS controlled 100% of the second instar larvae of this insect within 13 hours after application of the solution, proving to be more efficient than aqueous extracts of leaves and branches of cinnamon and tobacco powder, which showed a positive effect on the control of *P. xylostella* larvae only from the second, fifth and sixth day, respectively, after application (Dequech et al. 2009).

Despite the hypotheses raised above, we still do not know the mechanisms of action of the SbS. Thus, we believe that future research related to this topic should be carried out to better elucidate how this solution acts when in contact with *P. xylostella*. However, the product developed in the present study is a highly promising alternative to be used to control and minimize the damage caused by *P. xylostella* to Brassicaceae crops, since it has a low production cost, easy application, and handling, and also to be less harmful to the environment and human health when compared to traditional methods of control. Still, of the three concentrations tested in this study, the SbS at 1.25% was the one that we considered the most efficient for the control of *P. xylostella*, mainly of the observed biological parameters.

Conclusion

Based on the data obtained in the present study, it can be concluded that when applied to *P. xylostella*, the SbS controlled 100% of the individuals in just 13 hours after exposure, proving the effectiveness of the product. Regarding the ingestion test, no significant values were observed regarding larval mortality when leaves treated with the SbS were offered for feeding the young phase of *P. xylostella*. However, it was observed that this solution interfered in the

reproductive cycle of these species since it reduced the number of eggs, and had larvicidal and pupicidal action. So, the product developed here can be used as a new control tool against *P. xylostella* which is less harmful to the environment and human health when compared to synthetic pesticides.

Declarations

CRedit authorship contribution statement. Alini de Almeida: Data curation, Formal analysis, Investigation, Methodology, review & amp, editing, Writing – original draft. Edinéia P. S. Schmitz: Methodology, Writing – review & amp, editing. Gisele Louro Peres: Conceptualization, Funding acquisition, Methodology, Writing – review & amp, editing. Adelita Maria Linzmeier: Conceptualization, Funding acquisition, Methodology, Writing – review & amp, editing.

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Competing Interests. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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