Evaluation of *Bacillus sphaericus* bioinsecticide produced with white soybean meal as culture medium for the control of *Culex (Culex) quinquefasciatus*

Avaliação do bioinseticida de *Bacillus sphaericus*, produzido com meio de cultivo de farelo branco de soja no controle de *Culex (Culex) quinquefasciatus*

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Abstract

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Bioinsecticides are shown to be useful in control programs to prevent several diseases, based on their specificity and efficiency against insect vectors. In the current study a bioinsecticide based on Bacillus sphaericus *was produced using a white soybean culture medium and applied to larvae of* Culex quinquefasciatus, *the susceptible species, and* Aedes aegypti, *the refractory species used as the negative control. Efficacy was compared with that of the product fermented with the Luria Bertani (LB) reference medium. The experiments showed that* C. quinquefasciatus *was highly susceptible to the product prepared with white soybean meal, reaching 100% larval mortality even at 10mg/L, while* A. aegypti *failed to reach 70% mortality at a concentration of 1g/L. By comparison with the reference medium, the proposed culture medium showed high larvicidal power, reaching a LD90 of 2.26mg/L, while 4.37mg/L was needed for the LB medium to achieve the same mortality rate. Cost comparison between the formulations favored the use of the bioinsecticide produced with white soybean meal. After factoring in the LD90 value, the cost ratio favored the new raw material by nearly 1:220.*

Insecticides; Vector Control; Soybeans

Mosquitoes from the Culicidae family cause various kinds of direct and indirect harm to public health worldwide. In addition to bothering and irritating humans and other animals, provoking a drop in work productivity and livestock production, many species transmit pathogenic agents. In humans alone, microorganisms that cause serious diseases like malaria, dengue, yellow fever, filariasis, and arbovirus infections are transmitted during the blood meals of these insects, resulting in periodic outbreaks in the populations of various countries [1,2,3]. One of the principal factors in the extensive dissemination of some of these insects is their high capacity to reproduce in urban environments, acquired during their long history of co-evolution with the human species. This potential is clearly observed in *Aedes (Stegomyia) aegypti* Linnaeus, 1762 and *Culex (Culex) quinquefasciatus* Say, 1823, both highly anthropophilic Culicidae species that reproduce mainly in artificial breeding sites [4].

For many years, campaigns to combat mosquitoes were based on spraying chemical insecticides both to control larvae and kill adult insects. The initial results of this strategy proved quite satisfactory, leading to the control of various culicid-borne epidemics, especially in the early 20th century [5]. However, after several decades, the consequences of this approach proved quite harmful. Due to the insecticides' high residual power, the persistence of chemical compounds in the environment affects the entire food chain,

reaching humans themselves [6]. Over time, mosquitoes also developed resistance to chemical insecticides, thus undermining this control strategy's efficacy. Therefore, in recent years the use of organophosphate and organochlorine insecticides has been reduced, while their efficiency has been challenged [7,8]. Within this scenario, biological control based on endotoxin-producing bacteria like *Bacillus thuringiensis* var. *israelensis* Barjac, 1978 and *B. sphaericus* Neide, 1904, has proven effective in controlling insect vectors. These bacilli have the advantage of being safe to non-target organisms and non-accumulative in the food chain [9], and are thus considered environmentally safe by the World Health Organization (WHO) [10].

B. sphaericus is an aerobic bacteria that produces a round spore in the terminal portion of its cell. Two types of proteins, crystal toxins and Mtx toxins, produce the larvicidal effect by acting on specific receptors in the midgut of culicid larvae, causing a lethal cytopathological effect [11]. Despite its high toxicity, *B. sphaericus* is very specific, affecting only a small number of susceptible species [12]. Another important factor is the bioinsecticide's capacity to recycle itself in dead Culicidae larvae, resulting in greater persistence and greater larvicidal effect by the product [13]. Based on such characteristics, *B. sphaericus* bioinsecticides are indicated for combating lymphatic filariasis, West Nile fever, [3] and malaria [14], among other diseases.

In fermentations, *B. sphaericus* is considered an undemanding bacterial species, not requiring differential or selective culture media. Neither does it depend on special conditions to develop. Studies have already shown that a conventional aeration system is sufficient to satisfactorily ensure a good growth rate, sporulation, and formation of larvicidal toxin [15].

Still, although *B. sphaericus* is cultured using a simple technique, common to other microorganisms, the Luria Bertani (LB) medium reported in the literature for producing the bacteria is relatively expensive, since it requires high-cost laboratory ingredients. This leads to a significant increase in the bioinsecticide's cost, acting as a disincentive to its use on a large scale. In order to reduce the production costs and make the product more accessible to countries that suffer the most from culicid-borne diseases, several authors have suggested implementing local production of bioinsecticides, prioritizing the use of readily available, low-cost local substances [16,17,18].

Around the world, various agricultural products used to culture *B. sphaericus* have been tested successfully, based on seeds, vegetable extracts, and animal products [16,17,18]. In addition to the nutritional qualities needed for bacterial growth, the raw material must be widely available and cannot require any sophisticated or high-cost extraction or purification process, which would increase the product's end price and reduce its feasibility [15].

In the current study, we chose to produce a *B. sphaericus* bioinsecticide using a culture medium with white soybean meal as the base. The bioinsecticide was applied to L4 larvae of *C. quinquefasciatus*, the most susceptible species to the bacteria, and *A. aegypti,* the most resistant species, used as the negative control. The product's performance was assessed using mean larval mortality in a bioassay, together with the final price of the bioinsecticide's raw materials compared to the LB reference medium.

Material and methods

Bioinsecticide production

The *B. sphaericus* reference strain 2362 was kindly provided by Dr. Christina Nielsen Le-Roux of the Pasteur Institute, Paris, France.

The culture medium proposed in the current study was prepared with 40g/L of white soybean meal, a soybean byproduct widely used as a supplement in animal feed. The soybean cake was initially ground in a grinder and separated with a sieve with 35-mesh granulometry. Distilled water was added to the medium and the pH was adjusted to 7.0 adding a 10% NaOH solution.

The LB culture medium was prepared according to the usual formula published in the literature [19] for *B. sphaericus* fermentations. Briefly, the medium consists of peptone, yeast extract, and sodium chloride at a proportion of 2:1:2. In the current study, distilled water was added to the medium and the pH was adjusted to 7.2 by adding a 10% HCl solution.

Using lyophilized samples of *B. sphaericus* (strain 2362), the microorganism was inoculated in the LB medium and fermented at 30ºC for 24 hours. It was then divided into small aliquots and stored in a freezer at -4ºC, with 10% glycerin at a proportion of 1/3. The flask that was to serve as the inoculum consisted of 150mL of the culture medium, either LB or soybean meal, fermented for 24 hours at 30ºC. Next, the fermentations were performed in a 2L lab bench bioreactor, which received a volume of 1.5L. For each preparation, the media were sterilized in an autoclave at 120ºC for 20 minutes. The volumes were shaken at 200rpm, with an aeration rate of 1v of air per volume of medium. For the soybean meal culture medium, 2mL of polyethylene glycol were added

and the fermentation lasted 72 hours. The LB medium fermentation did not receive any anti-foaming agent, and also lasted 72 hours. For the current study, the fermentations were repeated three times on different days to ensure greater data accuracy.

Mosquitoes

A. aegypti and *C. quinquefasciatus* larvae were obtained from colonies maintained in the Molecular Parasitology Laboratory at the Federal University in Paraná, Paraná State, Brazil. *A. aegypti* eggs came from a reference strain from the Pasteur Institute and *C. quinquefasciatus* larvae were from the Brazilian Army Institute, Rio de Janeiro, Brazil. For rearing the colonies, the adults were kept in cages (25cm x 25cm) coved with screens and maintained in an environmental chamber at 28ºC. The adults were fed with an apple slice approximately 2cm2 thick and 4cm long, changed weekly. The blood meals also occurred weekly, using newborn mice. *A. aegypti* oviposition was done on filter papers inserted inside recipients with water (6cm x 6cm). The larvae were maintained in plastic trays (10cm x 25cm) with approximately 400mL of dechlorinated water, changed every three days. They were fed fish food *ad libitum* (Alcon Ind. e Com. de Alimentos Desidratados Alcon Ltda., Camboriú, Brazil).

Bioassays

The bioassays were performed in the same laboratory, based on the model recommended by WHO for experiments with the bacteria [20].

The tests took place in plastic recipients 10cm wide by 10cm high, containing a total of twenty 4th-instar larvae and 90mL of dechlorinated water. The volume was completed to 100mL by adding 10mL of the diluted bioinsecticide. The different concentrations of bioinsecticide were obtained from 1g of the recently fermented product, which was weighed and received volumes of water according to the scheduled dilutions.

The first bioassay was to confirm the specificity of the *B. sphaericus* bioinsecticide's action, applying it to *C. quinquefasciatus* and *A. aegypti*. Seven different dilutions of the product were used for this purpose: 1g/L, 200mg/L, 100mg/L, 50mg/L, 20mg/L, 10mg/L, and 5mg/L. The second bioassay aimed to quantitatively compare the efficiency of the bioinsecticide produced with the medium made of white soybean meal and that of the LB reference medium. Five different dilutions of the product were used: 10mg/L, 4mg/L, 2mg/L, 1mg/L, and 0.5mg/L. The bioinsecticides were applied to the recipients containing the lar-

vae, and the larval mortality rates were measured after 48 hours. The tests were performed in triplicate for each dilution, and three control recipients were prepared simultaneously, that is, with the same size and amount of larvae, but without receiving any dose of the fermented product. All the containers received a feed supply, and at the end of the target period the dead larvae were counted. The larval mortality rate was calculated and corrected by the Abbott formula [21]. If larval mortality exceeded 20% in the control flasks, the assay was invalidated. In the second bioassay, the LD_{50} and LD_{90} values were calculated by sigmoid adjustment using the Origin 5.0 software (Microcal Software, Massachusetts, USA). To detect significant differences in the efficiency of the two culture media (LB and soybean meal), we used the Student's t test for each dilution. All the tests were performed with a 95% confidence interval, $\alpha = 0.05$.

Results and discussion

In the first test, at a concentration of 5mg/L, the bioinsecticide cultured in white soybean meal produced 87.4% mortality for *C. quinquefasciatus* larvae, and 100% at the other concentrations, as shown in Figure 1. The product failed to show a similar performance with *A. aegypti*, with a much weaker effect at all the dilutions. The efficiency was observed to be dose-dependent, but still much lower than with *C. quinquefasciatus*, confirming the product's specificity for the latter culicid.

During the second stage, the performance of the proposed bioinsecticide was compared to that of the reference medium from the literature. Tested in *C. quinquefasciatus* larvae, the mortality results for the products fermented with white soybean meal and LB varied according to the concentration (Figure 2).

Based on the findings, both formulations showed high efficiency at the first dilution, but the white soybean medium performed better starting at 4mg/L (Figure 2). The white soybean medium performed significantly better at the next three concentrations as compared to the LB medium (t test; p < 0.05). Likewise, the LD_{50} and LD_{90} clearly favored the white soybean medium, as shown in Table 1. When applied to *C. quinquefasciatus* L4 larvae, the bioinsecticide cultured in white soybean produced an LD_{50} of 0.90mg/L and an LD_{90} of 2.26mg/L.

The LB medium did not show a similar performance with *B. sphaericus* (Figure 2). Mean mortality of *C. quinquefasciatus* larvae only remained high in the first two dilutions of the

Figure 1

Mean mortality rate for *Culex quinquefasciatus* and *Aedes aegypti* L4 larvae using *Bacillus sphaericus* bioinsecticide (strain 2362) produced with white soybean medium. Vertical bars indicate 95% confidence interval.
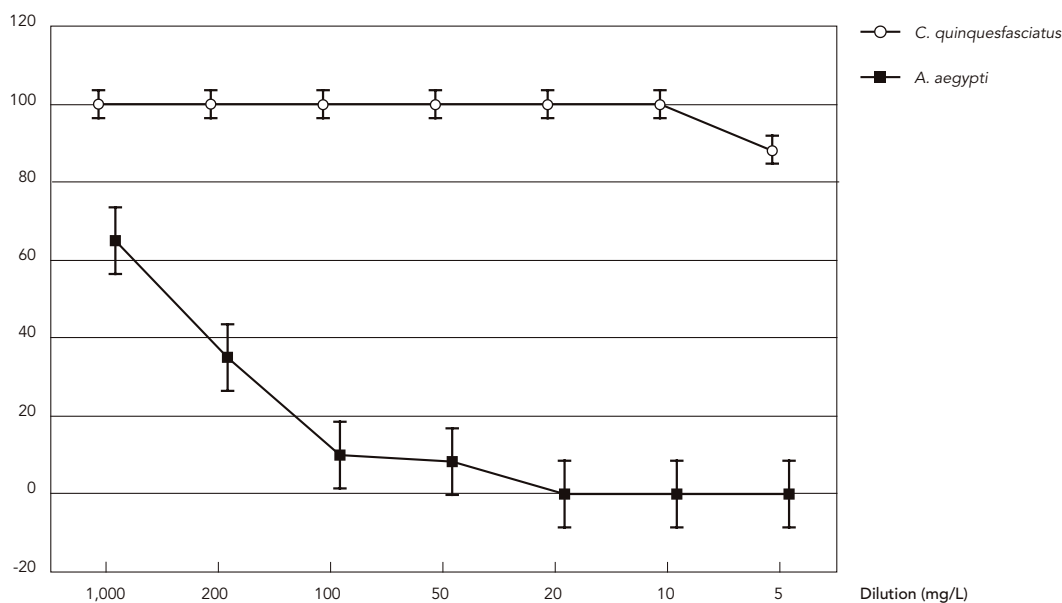
product, 10mg/L and 4mg/L. At a concentration of 2mg/L, we observed a drop in the bioinsecticide's toxicity, resulting in a mean mortality of 40.4%, which decreased even further until practically disappearing at 1mg/L. LD_{50} and LD_{90} for the *B. sphaericus* bioinsecticide produced with the LB medium were 2.43mg/L and 4.37mg/L, respectively.

The difference in the bioinsecticides' efficiency indicates better fermentation of *B. sphaericus* in the white soybean medium and thus higher production of endotoxin. This could be explained by the characteristics of the microorganism and the substrates present in the culture media. *B.*

sphaericus, unlike most microorganisms, does not use sugars in energy metabolism, because the bacteria lacks enzymes that are capable of metabolizing sugars as a source of carbon [22]. Alice et al. [23] succeeded in recreating this enzymatic function in *B. sphaericus* by inserting *Staphylococcus aureus*-specific genes, allowing the use of sugars in the bacterial growth. However, under normal conditions *B. sphaericus* is unable to process this type of substrate, so sugars are unnecessary in this bacteria's culture media. Poopathi et al. [24] tested a series of compounds for producing *B. sphaericus* and *B. thuringiensis* var. *israelensis*, standardizing a composition that used potato broth as the main component. Just as adding sugars is useless in the production of *B. sphaericus*, starch also proved ineffective in fermentations of this bacteria. Thus, the potato broth culture medium showed mortality results that were merely similar to those obtained with the LB medium. Energy compounds, saccharides, and polysaccharides are completely absent from the white soybean meal medium. On the other hand, the substrate contains high amounts of protein, characteristic of this soybean byproduct. The combination of the two characteristics proved highly favorable to *B. sphaericus* growth and endotoxin production.
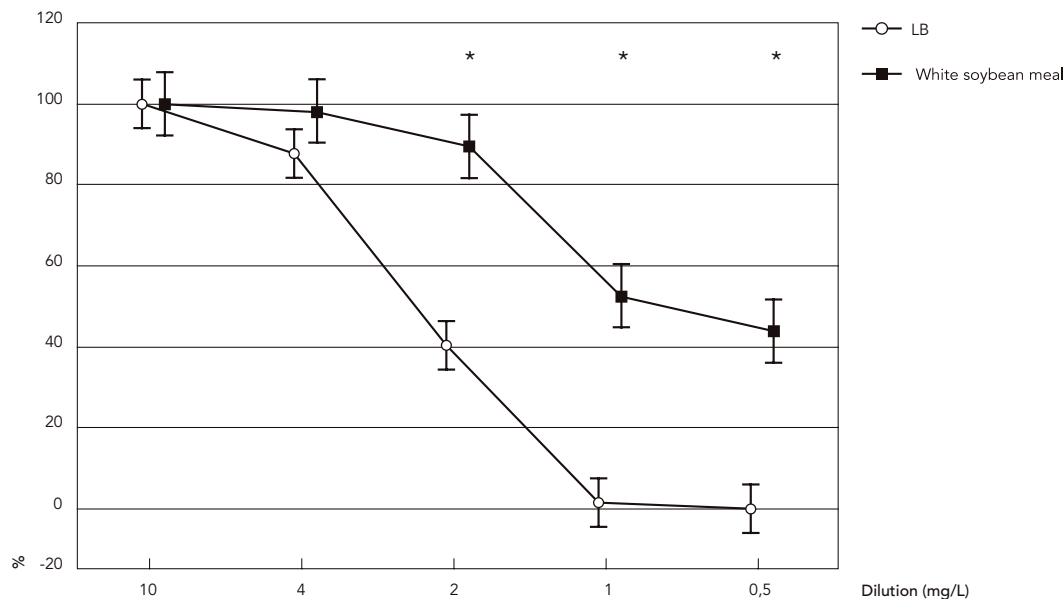
Table 1

Toxicity of *Bacillus sphaericus* bioinsecticide (strain 2362) produced with white soybean meal compared to Luria Bertani (LB) reference medium. Mean values, with maximum and minimum values in parentheses.

Culture medium	LD_{50} (mg/L)	LD_{90} (mg/L)	N
LB	2.43 (3.00-2.12)	4.37 (5.21-4.10)	6
White soybean meal	0.90 (1.08-0.76)	2.26 (2.36-2.00)	6

Figure 2

Mean mortality rate of *Culex quinquefasciatus* L4 larvae caused by *Bacillus sphaericus* (strain 2362) cultured in Luria Bertani (LB) and white soybean media. Vertical bars indicate 95% confidence interval, and asterisks mark significant differences between the media (t test; $p < 0.05$).

Just as the larval mortality rates clearly favored the bioinsecticide cultured with white soybean meal, the comparison proved even better when the cost of the ingredients was factored in, as shown in Table 2.

The difference in the cost of the two media was extremely disproportional due to the origin of the raw materials. While the LB reference medium is produced from high-cost laboratory substances sold in small amounts, the white soybean medium dispenses with such expensive ingredients. With white soybean meal as the principal component, the final cost is negligible, since this soybean byproduct is used in agribusiness as an animal feed supplement, sold only in bulk amounts. The cost of the raw materials used to produce 1L of the bioinsecticide with the LB medium is approximately US$ 7.87, while the same amount of medium produced with white soybean meal costs only US$ 0.07. The cost ratio for producing the culture medium is thus 1:110 in favor of the new bioinsecticide.

This ratio becomes even more unequal when considering the products' efficiency. Based on LD_{90}, it is estimated that approximately double the amount of product fermented with the LB medium would be needed to equal the mortality rate of the white soybean meal. The cost ratio would thus increase to 1:220.

Table 2

Cost comparison for producing Luria Bertani (LB) medium and white soybean medium.

Culture medium	Composition	Amounts (g/L)	Final cost (US$/L)	Cost difference for LD_{90}
LB	Peptone + yeast extract + sodium chloride	10 + 5 + 10	7.87	1:220
White soybean meal	White soybean meal + polyethylene glycol	40 + 2	0.07	-

Resumo

A utilização de bioinseticidas tem se mostrado útil aos programas de prevenção de diversas enfermidades devido a sua especificidade e eficiência contra insetos vetores. No presente trabalho, o bioinseticida de Bacillus sphaericus *foi produzido com um meio de cultivo composto de farelo branco de soja e aplicado em larvas de* Culex quinquefasciatus, *espécie suscetível, e* Aedes aegypti, *espécie refratária, usada como controle negativo. O desempenho foi comparado com o do produto fermentado com o meio referência Luria Bertani (LB). Os experimentos constataram que* C. quinquefasciatus *apresentou uma alta suscetibilidade ao produto produzido com farelo branco de soja, alcançando mortalidade de 100% mesmo na diluição de 10mg/L, enquanto* A. aegypti *não atingiu 70% na concentração de 1g/L. Quando comparado com o meio referência, a formulação proposta proporcionou um alto poder larvicida, alcançando uma DL_{90} de 2,26mg/L, ao passo que LB precisou de 4,37mg/L para a mesma mortalidade. A comparação do custo das formulações favoreceu o bioinseticida produzido com o meio farelo branco de soja. Considerando os valores de DL_{90}, a relação do custo das matérias-primas ficou próxima de 1:220.*

Inseticidas; Controle de Vetores; Soja

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