Entomopathogenic activity of *Lysinibacillus sphaericus* in field realistic dosages of glyphosate in *Aedes aegypti* and *Culex quinquefasciatus* larvae resistant to TEMEPHOS

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Resumen

Los herbicidas a base de glifosatos son ampliamente usados para el control de hierbas perennes alrededor del mundo. Este compuesto es altamente persistente en el ambiente, y tiende a filtrarse a cuerpos acuáticos, resultando en la afectación de organismos no objetivos, como por ejemplo larvas de mosquito. *A. aegypti* y *C. quinquefasciatus* son mosquitos vectores de diversos arbovirus como el dengue y la encefalitis equina, respectivamente. Por otro lado, *L. sphaericus* es una bacteria conocida por su capacidad larvicida y la metabolización del glifosato por medio de la vía CP- liasas que genera el ion ortofosfato y glicina. En el presente estudio, se busca determinar si la formulación de glifosato (Roundup ®) afecta de alguna manera la actividad entomopatógena de *L. sphaericus* en larvas de *C. quinquefasciatus* y *A. aegypti*. Para este fin, se realizaron múltiples bioensayos con bacteria, glifosato y la mezcla de estos compuestos; así como también se realizaron curvas de toxicidad (LD50). Dichos datos se analizaron con una ANOVA unifactorial y para determinar la LD50, se utilizó un ajuste al modelo probit. Se pudo observar un efecto sinérgico en la mortalidad de las larvas cuando se tenía *L. sphaericus* y glifosato en la mezcla. De igual forma, *C. quinquefasciatus* es más sensible a *L. sphaericus* que *A. aegypti*, por lo que observar el efecto sinérgico en esta especie es un poco más complicado que con *A. aegypti*. Se requieren estudios moleculares para determinar la producción de toxinas por *L. sphaericus* y así ganar más entendimiento sobre este proceso sinérgico.

Abstract

Glyphosate-based herbicides are one of the most commonly used compounds for the control of perennial weeds around the world. This compound is very persistent in the environment, and tents to filter onto the aquatic ecosystems, resulting in the affectation of non-target species such as mosquito larvae. *A. aegypti* and *C. quinquefasciatus* mosquitoes are vectors of multiple arboviruses such as Dengue, West Nile Virus and Zika. Besides, *L. sphaericus* is a bacterium capable of both degradation glyphosate into more environmentally friendly compounds and kill *A. aegypti* and *C. quinquefasciatus* larvae. In this study, we want to assess if glyphosate formulation on typically found concentrations of water affects in any way the entomopathogenic activity of *L. sphaericus* on *A. aegypti* and *C. quinquefasciatus* individuals. Bioassays and toxicity curves were performed to compare the larval mortality
between different treatments with and without bacteria and glyphosate (Roundup®), comparisons were made with both probit regression and ANOVA analysis. A synergic effect on larval mortality was found when *L. sphaericus* and glyphosate were mixed. *C. quinquefasciatus* is more sensitive to *L. sphaericus*, which makes difficult to note the synergic effect of glyphosate and *L. sphaericus* in comparison with *A. aegypti* larvae. Molecular studies focused on the toxin production of *L. sphaericus* are required to understand more about this synergy effect.

Keywords.

Glyphosate, *Lysinibacillus sphaericus*, *Aedes aegypti*, *Culex quinquefasciatus*, Entomopathogenic activity,

1. Background

Glyphosate-based herbicides are one of the most commonly used compounds for the control of perennial weeds around the world [1]. Traces of glyphosate and its main metabolite AMPA were found in drinking water and human urine from farmers in Mexico [2]. Furthermore, these compounds were also found in water from soybean crops in Argentina and water samples of Mideast USA [3,4]. Glyphosate and AMPA are very persistent compounds in the environment and are toxic to non-target organisms [5].

In Colombia the usage of glyphosate is not only centered in agriculture, in 1999 the “Plan Colombia” started the massive aerial aspersions of glyphosate to eliminate the illicit coca crops [6]. Which not only failed to eradicate the Coca crops but also endangered other non-target species in the process [6-9]. Due to this, the contamination of water ecosystems by glyphosate or AMPA is of great concern to the environment in Colombia.

Furthermore, little is known about glyphosate interaction with other organisms different from plants. Helbert., et al 2014 and Ríaz et al., 2009 demonstrated that glyphosate had an impact on small populations of bees and mosquito larvae, making them more susceptible to neurological damage and resistant to insecticides, respectively [10, 11]. This founding suggests that more investigation is needed to establish the effect of glyphosate and AMPA in the non-target species that have bigger effects of the trophic chains and in the public health concern.

In Colombia, *Aedes aegypti* is the principal vector of multiple arboviruses such as dengue, Zika, and chikungunya [12]. These mosquitoes are very important vectors due to their diurnal activity and preference for human blood. Additionally, *A. aegypti* larvae are found in water for both domestic and peri-domestic environments. Recently, the appearance of *A. aegypti* mosquitoes resistant to insecticides is growing [13]. Because of this, it is imperative to search for other strategies of eradication that does not affect both the human populations and the environment, for example, biological control.
On the other hand, *Culex quinquefasciatus* larvae are one of the most recognize vectors of West Nile virus [14]. In Colombia this mosquito can be found cohabiting with *A. aegypti* larvae, as well as in higher altitudes [15].

Many microorganisms are used as biological agents against plagues, for example, *Trichoderma harzianum* used as mycoparasite fungi against phytopathogenic species and *Bacillus thuringiensis* as an entomopathogenic bacteria applied in crops [16, 17]. Recently, Colombian strains of *Lysinibacillus sphaericus*, a well-known entomopathogenic gram-positive bacteria, have demonstrated strong activity against *Culex, Aedes*, and *Anopheles* larvae. [18, 19] *L. sphaericus* is not only a very effective bio-controller of said organisms, but it is also capable of removing toxic metals on water, promote plant growth and, metabolize glyphosate by a pathway that does not produce AMPA, but instead produces glycine and orthophosphate ion. [20 - 22].

In this study we want to asses if glyphosate formulation on typically found concentrations of water affects in any way the entomopathogenic activity of *L. sphaericus* on *A. aegypti* and *C. quinquefasciatus* larvae.

2. Material and Methods

2.1. *Lysinibacillus sphaericus* strains

*L. sphaericus* strains used in this study were the WHO reference strain 2362 and III(3)7, a Colombian strain isolated from a native oak tree (*Quercus humboldtii*) [23]. This mixture was determined by Rojas, et al (2018) as the most lethal for *A. aegypti* larvae [24].

2.2. *Aedes aegypti* and *Culex quinquefasciatus* maintenance

*A. aegypti* and *C. quinquefasciatus* third instar larvae were collected from La Mesa Cundinamarca (4°38'05.9"N, 74°27'45.4"W), a well-known area of Temephos resistance individuals. Larvae were kept at 28±0.03 °C and 70% relative humidity under 12:12 light/dark photoperiod.

2.3. Toxicity assays

To establish the Lethal Dose 50(LD50\textsubscript{2362+III(3)7}) of the bacterial mixture on the larvae, a toxicity curve was in order. In total five different concentrations of bacterial inoculum were used, 10^5 UFC/mL, 10^6 UFC/mL, 10^7 UFC/mL, 10^8 UFC/mL, 10^9 UFC/mL. 20 third instar larvae were placed into glass containers with 30 mL of chloride-free tap water. Larvae were kept at the same conditions as used in the bioassays. Finally, a probit regression was applied to establish the LD50.

2.4. Bioassays
**L. sphaericus** strains were grown in nutritive agar (Oxoid.Thermo Scientific™ Ref: CM0003) for 15 h at 30 °C. Cells were collected and resuspended in 10 mL of distilled sterile water followed by a series of dilutions to set the initial inoculum to $10^7$ UFC/mL.

Monsanto’s glyphosate formulation Roundup® 747 was used at an equivalent concentration of 1.69 g of glyphosate per litter. Colombian farmers use the entire package content of Roundup® 747 on 20 L of water.

The montage of all these bioassays was described in Rojas and Dussán [18]. In which, 20 A. *aegypti* or *C. quinquefasciatus* third instar larvae were placed into a glass flask containing 30 mL of chloride-free tap water. Pending on the treatment, 300 uL of bacterial suspension and glyphosate formulation was added. Treatments used in this study are described in table 1. Each treatment and control were tested in triplicate, and all bioassays were repeated thrice. Bioassay conditions were 28 ± 0.3 °C, 70% relative humidity and a 12/12-h light/ dark photoperiod. Larvae mortality was reported after 24 and 48 h of exposure, larvae with no response to physical stimuli or unable to attach to the surface were counted as dead.

### 2.5. Statistical analysis

All statistical tests were carried out with the R 3.1.2 statistical package (R Core Team 2015), and a level of significance of p < 0.05 was chosen for every test. To determinate the difference between mortality of larvae in each bioassay, an ANOVA test was performed.

### 3. Results and discussion

#### 3.1. LD50

A lethal dose 50 for the mixture of *L sphaericus* 2362 and III(3)7 was found to be $10^7$ UFC/mL for *A. aegypti* larvae and $10^4$ UFC/mL for *C. quinquefasciatus*. This dosage turned out to be the same as the one published in 2018 by Rojas et al [24]. Given these results, all bioassays were calibrated to that concentration. After the measurements of larval mortality, we observed that at the LD50 gly for is of 2.34 g/L. This result allows us to use the field-realistic concentrations of glyphosate (1.69 g/L). Furthermore, as far as our knowledge goes, the maximum concentration tested on this species was for 0.2 g/L [25] which is 10 times less the concentration used on this study. With that information, we can assume that the tolerance of the larvae of La Mesa -Cundinamarca to glyphosate is due to the exposition to higher concentrations of this molecule and other organophosphate compounds. This last information is not only interesting for this study, but it also reflects the resistance to insecticides and the real exposition of this non-target species to the indiscriminative use of both insecticides and glyphosate in the Colombian rural areas.

#### 3.2. Entomopathogenic activity of *L. sphaericus* on glyphosate

For *A. aegypti* larvae, a significative difference in the mortality of the larvae exposed to glyphosate and the bacterial mixture compared to the treatments on the same measurements
of time was observed (Fig. 1). At 24 hours, the difference in the mortality between the treatments and the bacterial mixture with glyphosate was about four times the observed (ANOVA: p-value $2 \times 10^{-16}$, $F = 67.87$; average larvae mortality of bacterial mixture at 24 h: 20%, average mortality of glyphosate at 24h: 20%; average mortality glyphosate and bacterial mixture at 24h: 80%).

Correspondingly, at 48 h, the differences in larval mortality between treatments with L. sphaericus and glyphosate and the others were highly marked (ANOVA p-value = $7.87 \times 10^{-14}$, $F = 47.37$).

These results may indicate that the addition of glyphosate to L. sphaericus can produce a synergic effect on the larvicidal activity. To verify if the bacterial communities were affected by glyphosate Roundup® addition, plating assays were performed at 0, 24 and 48 hours (Fig. 2). In this case, the bacterial titer was not changed, yet the sporulation was faster on those assays that had glyphosate (Data not shown).

In this study, we found a possible synergetic behavior on the larvae mortality when glyphosate (Roundup®) was added to the bacterial mixture. As reported by González and Dussán (2018), the sporulation was stimulated by the glyphosate addition [20]. These results are intriguing to observe because A. aegypti larvae are immune to toxins Bin A/B produced primarily on the sporulation process of L. sphaericus [26]. Thus, the expected reaction would be to see a decay onto the larvae mortality once the sporulation process of the bacteria is completed in no more than 9 hours, yet, our results show a different behavior.

This evidence drives us to generate two hypotheses, the first one is that, in Roundup® formulation there is a particularly toxic surfactant use to increase glyphosate function, polyethoxylated tallow amine (POEA), multiple reports affirm that this compound induced DNA damage onto embryos of zebrafish (Danio rerio) and was lethal to all the aquatic species of both bacteria, algae, and amphibians [26, 27]. In our study given the average mortality presented onto the treatments with only glyphosate (Fig 1), we conclude that mortality on larvae can be partly assigned to the adjuvants of Roundup® formulation, yet there could be an interaction between the adjuvants and L. sphaericus, that dramatically increases the toxicity onto the larvae. To prove that, more in-depth studies are required.

Our second hypothesis is that, given the evidence presented before, L. sphaericus has the capability of degrading glyphosate onto two main molecules, glycine, and orthophosphoric ion [20]. Those two molecules can be easily used by both the larvae and the bacteria in different metabolic pathways such as phosphorylation of proteins. Increasing the overexpression of different proteins such as the Mtx1-3, chitinase, and the S layer protein also increases the larvicidal activity against A. aegypti larvae, which can explain the difference between the mortality when L. sphaericus and glyphosate are alone than in mixture [26,29].
A pilot to test whether if the glyphosate and its two main degradation intermediates were the explanation on the larvae mortality when used in the mixture. A small assay using the equivalent concentration of orthophosphate ion (data not shown) as if it was the result of glyphosate degradation by *L. sphaericus*. Interestingly a similar pattern on the larvae mortality was observed when added de bacteria mixture and the orthophosphate ion, larvae mortality drastically augmented (Data not shown).

For *C. quinquefasciatus* larvae, significative differences were noted between the mortality of the treatments that had *L. sphaericus* and the others (Fig. 3 p-values < 0.00001, F = 28.59 at 24h and 48h p-values < 0.00001, F = 20.17). *C. quinquefasciatus* is more sensitive to *L. sphaericus*, which makes difficult to note the synergic effect of glyphosate and *L. sphaericus* as it was with *A. aegypti* larvae. This evidence suggests that glyphosate does not impair the entomopathogenic activity of *L. sphaericus*, and as a contrast, can potentiate it. No changes to the bacterial titter were noticed (Fig 4).

4. Conclusions

A significative difference between larvae mortality on treatments exposed to both glyphosate formulation and *L. sphaericus* mixture was found. It is needed to measure the levels of glyphosate, AMPA, and glycine to clarify if the larvae mortality is mediated by the metabolism of glyphosate by *L. sphaericus* or by the effect of the adjuvant agents onto both larvae and bacteria.

Similarly, it is required to study the production of toxins from *L. sphaericus* to determinate when exposed to glyphosate, glycine or orthophosphoric ion, which are metabolites produced by the cp-lyase pathway used by *L. sphaericus* to degrade glyphosate. As well as also needed to explore the production of toxins when exposed to the POEA adjuvant present onto the Roundup® formulation to observe the impact as well on the entomopathogenic activity of this bacteria.

Finally, the larval tolerance to glyphosate is of both great concern as well as intriguing due to the capacity of *L. sphaericus* to control those populations of mosquitoes so resistant to different toxic compounds. These results show clearly the need to study the present conditions of these vectors as well as the implementation of plague management plans that evades the use of toxic compounds.

5. Acknowledges

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References


### 7. Table and Figures

<table>
<thead>
<tr>
<th>Treatments denomination</th>
<th><em>A. aegypti</em> or <em>C. quinquefasciatus</em> larvae</th>
<th><em>L. sphaericus</em> (2362 +III(3)7)</th>
<th>Glyphosate 1.69g/L</th>
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<tr>
<td>Control</td>
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<tr>
<td>Larvae + (2362 + III(3)7)</td>
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<td>Larvae + 2362+III(3)7 + Glyphosate</td>
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Table 1. Description of treatments implemented in this study. (+) signifies added, (-) not added. *L. sphaericus* strains were 2362 and III(3)7.

![Fig 1. Larvae mortality onto the different treatments. Boxes represent quartile range, crosses inside the plot represents the media of the assays. Significative differences between Glyphosate+2362+III(3)7 and the other treatments onto the same time of larvae mortality 24 and 48 h was found (p-value < 0.00001, represented as *** onto the graphic). Additionally, between the control and the treatments with only bacteria or glyphosate were significatively different (P-value< 0.007, represented as ** onto the graphic). No significative differences were found between the treatment of only bacteria and glyphosate.](image)
Fig 2. Plating assays of *L. sphaericus* mixture on the different treatments at tree times 0, 24, 48 h for the *A. aegypti* larvae.

Fig 3. *Culex quinquefasciatus* larvae mortality onto the different treatments. Boxes represent quartile range, crosses inside the plot represents the media of the assays. Significative differences between treatments with *L. sphaericus* onto the same time of 24 and 48 h was found (p-value < 0.00001, represented as *** onto the graphic). Additionally, not significatively differences were found between the control and the glyphosate (P-value > 0.05) . At 48 h difference on larvae mortality were found between the glyphosate and the control (P value < 0.003, represented as * onto the graphic).
Fig 4. Plating assays of *L. sphaericus* mixture on the different treatments at tree times 0, 24, 48 h for the *C. quinquefasciatus* larvae.