Colegio de Postgraduados

Glyphosate-, carbofuran-, and chlorpyrifos-tolerant *Priestia aryabhattai*, isolated from agricultural soils

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ABSTRACT
Objective: To isolate and carry out a molecular characterization of microorganisms potentially tolerant to high concentrations of different pesticides (glyphosate, carbofuran, and chlorpyrifos).
Design/Methodology/Approach: Based on the isolate project SIP20170193, only the strain GVE 5 was chosen for the experiment, as a result of its morphological and growth characteristics. The tolerance capacity (TC) of GVE 5 to three different pesticides (glyphosate, carbofuran, and chlorpyrifos) was evaluated in two different media (LB and M9). The only carbon source was 200 mg/L of each pesticide. GVE 5 was identified through the Polymerase Chain Reaction (PCR) molecular techniques and amplified by the 16S rRNA marker.
Results: Based on the TC analysis, the GVE 5 strain of *Priestia aryabhattai* recorded a growth with 200 mg L^{-1} of glyphosate, carbofuran, and chlorpyrifos in LB medium and M9 minimal medium.
Study Limitations/Implications: There were no limitations or implications for this study.
Findings/Conclusions: *Priestia aryabhattai* is tolerant to 200 mg L^{-1} of glyphosate, carbofuran, and chlorpyrifos. These results open new lines of research regarding the bioremediation of soils polluted by these agrochemicals. *Priestia aryabhattai* should be subjected to further evaluations as a plant growth promoter.

Keywords: pesticides, tolerance, *Priestia aryabhattai*.

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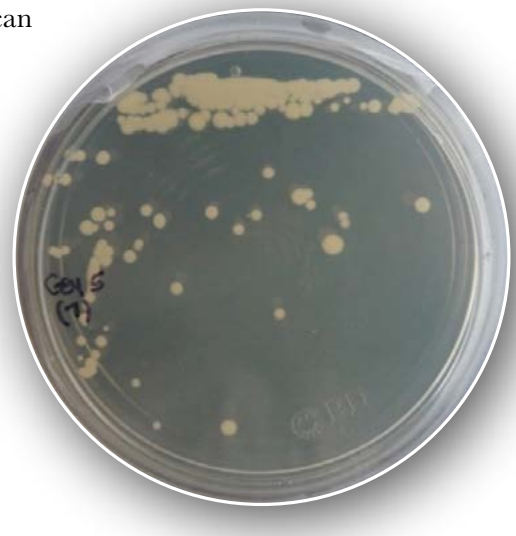
INTRODUCTION

The competition against weeds and pests can impact the quality and yield of food crops in agricultural systems. Throughout the world, agroecosystem sustainability is seriously threatened by the widespread use and release of several agrochemicals. In Mexico, pesticide production volume reached 59,157 t in 2017 (INEGI, 2018). For their part, Sinaloa producers use about 30% of the total pesticides applied in northeastern Mexico (Leyva *et al.*, 2014). Currently, the use of 183 active ingredients (AI) of highly



hazardous pesticides (HHP) has been authorized. Out of this total, 33% AI are included in the Official Pesticide Catalog (CICOPLAFEST 2016). If inhaled, these AI can be extremely dangerous and deadly. In addition, they are considered as probably carcinogen, carcinogen, mutagenic, bio-accumulative, persistent in water, soil, or sediments, and very toxic to aquatic organisms and bees.

The accumulation of these pesticides in the environment through various processes can reduce soil capacity, impacting biological production functions, environmental protection, and human health (Vallejo, 2013). Microorganisms can biodegrade pesticides and their metabolites from the environment, while microbial consortium can completely biodegrade them (Ortiz *et al.*, 2013). The capacity of microorganisms to tolerate and degrade pollutants is associated with their long-term adaptation to highly polluted environments. Commonly identified microbes used for bioremediation purposes against pesticides are: *Pseudomonas* spp., *Bacillus* spp., *Klebsiella* spp., *Pandoraea* spp., *Phanerochaete chrysosporium*, and *Mycobacterium* spp. (Odukkathil and Vasudevan, 2013). As a result of their biochemical capacity to adapt to the environment, bacteria can easily induce mutant strains. Consequently, further studies are required about this subject (Racke *et al.*, 1996; Oliveira *et al.*, 2015). During the last few years, efforts have been made to increase the sustainability of agriculture, seeking biological solutions to degrade pesticides. Therefore, the objective of this study was to isolate, identify, and characterize the molecules of bacteria that can tolerate a 200 mg L^{-1} concentration of glyphosate, carbofuran, and chlorpyrifos, under in vitro conditions. The aim was to determine a consortium and to develop a potential biotechnological catalog for the bioremediation of soils impacted by these agrochemicals. In the future, these microbial consortia could also have an additional purpose: to play a role in plant growth promotion.

MATERIALS AND METHODS

Chemical product

The commercial brands FAENA® (35.6% glyphosate), Furadan® (350L of carbofuran), and CHLORBAN® (480EC of ethyl chlorpyrifos) were used to evaluate tolerance in the M9 minimal medium.

Strain and pesticide selection and hemolytic activity assessment

Based on the results from the SIP20170193 project, a fast-growing strain (GVE 5) was chosen. GVE 5 came from Guasave, a region where potato crops dominate agricultural systems. The production or lack of hemolysis was determined in 5% blood agar from a bovine blood serum. Striatal-inoculation was carried out and, subsequently, the product was incubated at 28 °C for 18 h (Haubert *et al.*, 2017). A pesticide database was developed to determine the agrochemicals, taking into account family, degradation processes, and half-life (DT50), based on the following sources: TOXNET (https://toxnet.nlm.nih.gov), PubChem (https://pubchem.ncbi.nlm.nih.gov), and DISI (https://disi.gob.mx/agroquimicos/).

Evaluation of the tolerance capacity to 200 mg/L glyphosate, carbofuran, and chlorpyrifos

A M9 minimal medium was prepared: $Na_2HPO_4 \cdot 7H_2O$ 48 mM, KH_2PO_4 22 mM, NaCl 8.6 mM, NH_4Cl 18.7 mM, $MgSO_4$ 1 mM, and $CaCl_2$ 0.1 mM: pH 7-7.2 (adjusting HCL 1 M NaOH 5 M) (Graf and Altenbuchner, 2011; Shabbir *et al.*, 2018)II and III. Once the medium was sterilized, 200 mg L^{-1} of a commercial pesticide pattern (glyphosate, carbofuran, and chlorpyrifos) were added at 40 °C as the only source of carbon. A 2 g L^{-1} stock of each pesticide was dissolved in sterile deionized water. Subsequently, it was filtered in a 0.22 μm acrodisc and kept at 4 °C until it was applied with striatal Kolle handles. Afterwards, it was incubated at 28 °C for seven days, using a modified version of the methodology proposed by Shabbir *et al.* (2018). A blank and a control were used in the experiment to guarantee the reliability of the results and the control measures, as well as to obtain statistically significant data for the four repetitions.

Sanger sequencing molecular characterization: DNA extraction and PCR

Once the GVE 5 strain had been reactivated and purified, a repeated subculturing in stock was carried out with each strain, in order to obtain the biomass. Subsequently, the DNA was extracted according to the instructions of the DNeasy UltraClean Microbial Kit QIAGEN. Afterwards, the DNA was visualized in 1% agarose gel, stained with ethidium bromide. The 16S1 (AAGGAGGTGATCCAGCC) and 16S2 (GAGASTTTGATCHTGGTCAG) primers were used to amplify the areas preserved in 16S. A final volume of 25 μL was obtained, preparing the following amplification reactions: 5 μL DNA [20 ng], 0.5 μL of each oligonucleotide [10 μM], 5 μL buffer PCR [5X], 2 μL of $MgSO_4$ [25 mM], 0.5 μL of dNTP's [10 mM], 0.2 μL of Taq polymerase [5 U/μL], and ULTRAPURA water. The reactions were placed in a thermocycler, under the following conditions: initial denaturation at 95 °C for 4 min; denaturation at 95 °C for 1 min; alignment at 70 °C for 1 min; extension at 72 °C for 2 min, and final extension at 75 °C for 5 min. Afterwards, the PCR products were processed in an electrophoresis chamber, with a 1% agarose gel, at 90 V, during 35 min (Sambrook and Russell, 2001). The results were observed in a photodocumenter, under ultraviolet light. The PureLink™ PCR Purification Kit Invitrogen was used to purify the PCR products, following the instructions provided by the manufacturer.

Sequencing and phylogenetic analysis of 16S rRNA

A NanoDrop™ spectrophotometer was used to measure the purity and concentration of the PCR product. PCR products that complied with the specifications were sent to the Instituto de Biotecnología of the UNAM, where they were subjected to a Sanger sequencing, and to the MACROGEN company, where the FASTA archives and other quality archives were developed, using the CONSED packaging. The resulting sequences were analyzed with the BLAST program and the results were compared with those published by the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). Subsequently, the MAFFT software (Katoh, 2002) was used to carry out a multiple alignment and the GBlocks software was used to filter them. GBlocks selects the

preserved blocks of multiple alignments to use in phylogenetic analysis. Once the analysis was completed, the Model Test software (Posada, 2006) identified the substitution model. The Silva database (https://www.arb-silva.de/aligner/) and the RAXML lab (Maximum likelihood) were used to build the phylogenetic trees.

RESULTS AND DISCUSSION

In order to purify the strain and to establish the morphological characterization, the assay, and the molecular identification, the GVE 5 strain was reactivated in the LB medium. These stick-shaped and aerobic bacterium is gram-positive, forms spore, has a negative hemolysis, and its optimal growth range is 28-37 °C. Based on these phenotypical characteristics, it can be classified as a mesophyll organism (Figure 1). This research also established its tolerance to 200 mg L^{-1} of glyphosate, carbofuran, and chlorpyrifos, in two different culture media (LB and M9). Figure 2 shows the assays that determined the GVE 5 growth for each pesticide. The results indicate that this strain could have a biochemical arsenal that could degrade these organic compounds, despite their capacity to persist in the environment. The molecular characterization continued at the 16S rRNA marker level. The PCR molecular technique was used for amplification purposes, obtaining a ≈1,500 pb area, which was purified and subsequently subjected to a sequencing process (Figure 3). Therefore, the genus *Priestia* predominated and the GVE 5 strain was identified as *Priestia aryabhattai*. The substitution analysis per area showed a 0.010 nucleotide distance (Figure 4). Narsing Rao *et al.* (2019) evaluated *Bacillus aryabhattai* and proposed its reclassification as an a posteriori heterotypic synonym of *Bacillus megaterium* de Bary 1884 (Approved lists 1980). Based on the conserved signature indels (CSI), Gupta *et al.* (2020) proved in October 2020 that many *Bacillus species* (including the *Subtilis* and *Cereus* clades) make up a total of new 17 individual clades. The authors proposed acknowledging these clades as new genus. In addition, they suggested naming the *Megaterium* clade as *Priestia* gen. nov., because it includes all the old *Bacillus* species: *B. megaterium*, *B. abyssalis*, *B. aryabhattai*, *B. endophyticus*, *B. filamentosus*, *B. flexus*, and *B. koreensis*. This situation is the result of the two CSI included in the oligoribonuclease (NrnB) that were shared by all the members of the clade (Gupta *et al.*, 2020). During the last years, the beneficial effect of *P. megaterium* on plant growth has

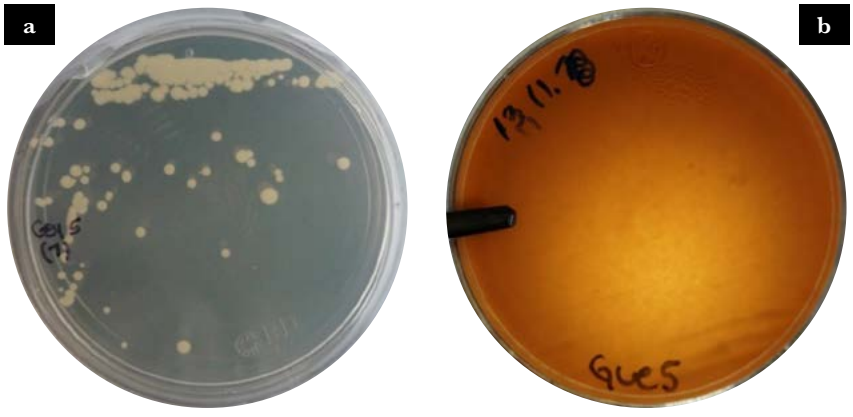


Figure 1. *Priestia aryabhattai* bacterium (GVE 5) (a) Morphology in LB agar (b) Blood agar hemolysis test.

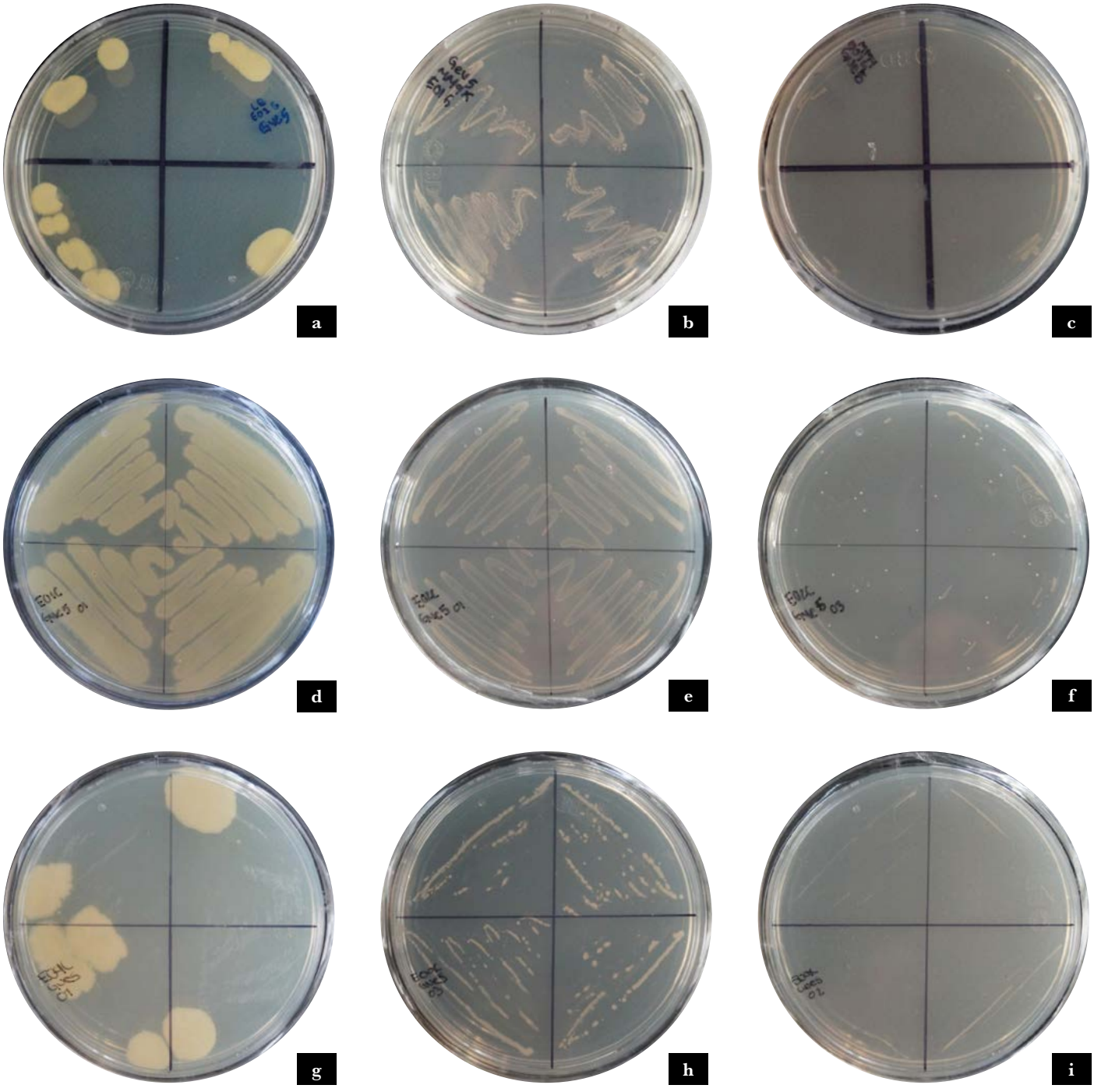


Figure 2. Glyphosate, carbofuran, and chlorpyrifos tolerance test. *Priestia aryabhattai* bacterium (GVE 5). LB medium 200 mg L^{-1} of the pesticide pattern of a commercial brand: glyphosate (a), carbofuran (d), and chlorpyrifos (g). M9 minimal medium 200 mg L^{-1} of the pesticide pattern of a commercial brand: glyphosate (a), carbofuran (d), and chlorpyrifos (g). M9 minimal medium without pesticide pattern (c), (f), and (i).

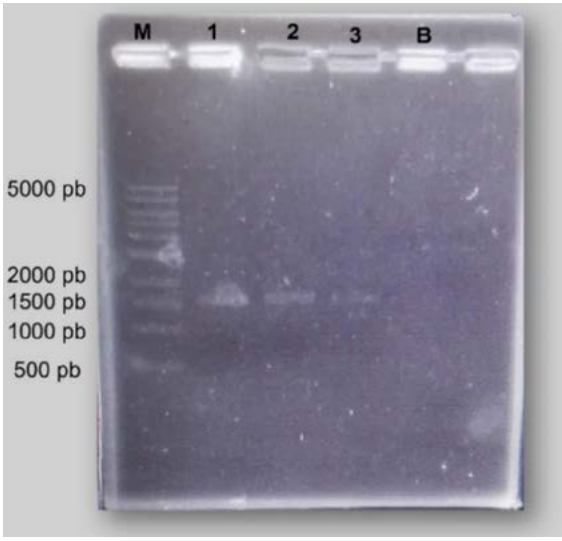


Figure 3. Electrophoresis results in 1% agarose gel (PCR product). Column (M): 500 pb DNA molecular weight marker, 200 lanes, columns (1), (2), and (3) GVE 5 strain, column (B) negative control.

Figure 4. Cladogram of the nucleotide sequencing alignment (GVE 5).

increasingly become a subject of interest (Biedendieck *et al*., 2021). In addition, Elarabi *et al*. (2020) isolated a strain that can tolerate 50, 100, 150, 200, and 250 mg mL^{-1} maximum concentrations of glyphosate. The evaluation was carried out using a mineral salt medium (MSM), quantifying the colony forming units (CFU) during seven days. The strain was identified as *Bacillus aryabhattai* FACU3 by 16S rRNA.

CONCLUSIONS

Priestia aryabhattai can tolerate 200 mg/L of glyphosate, carbofuran, and chlorpyrifos. Its TC was proved through the growth assay carried out in M9 minimal medium, taking into consideration the pesticide pattern as the only source of carbon. In addition, the 16S rRNA marker was used for molecular identification. In conclusion, *Priestia aryabhattai*

should be not just included in a biotechnological catalog for the bioremediation of soils polluted with these agrochemicals, but also should be subjected to further evaluations as a plant growth promoter.

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