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BACTERIAL ISOLATIONS OF PLOTS WITH DIFFERENT AGRICULTURAL MANAGEMENT, METABOLIC EVALUATION AND VIABILITY WITH DIFFERENT PESTICIDES

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ABSTRACT

Bacteria are organisms that are of great importance in agriculture since they contribute in multiple processes related to the availability of nutrients in the soil, degradation of organic matter, control of some pathogens, degradation of toxic compounds, among others. This is because of the great diversity of existing bacteria that result in a wide range of components that can metabolize. The objective was to evaluate the metabolic processes in soil bacterial isolates and observe the inhibition of these isolates in the presence of Furadan, Esteron and Malathion. The strains obtained were isolated from soil samples from plots with conventional management and plots that tend to agroecological management, the isolates were identified, they underwent the metabolic test in presence of different substrates, the biofilm formation capacity was evaluated and their viability was evaluated in presence of Furadan, Esteron and Malathion. *Pseudomonas* and coliform genera were identified, the isolates from conventional management samples were more activity in presence of different substrates, and they had a greater capacity to form biofilm, and had lower bacterial inhibition in presence of the three pesticides evaluated. When comparing the convention management samples and the agroecological management samples, those that had the best metabolic activity against different substrates, biofilm formation and less inhibition against the three pesticides evaluated were those of conventional management.

Keywords: biofilm; bacterial inhibition; metabolism; soil; substrates.

INTRODUCTION

Agrosystems have different types of management; among these are agroecological management and conventional management. Agro-ecological management is based on agriculture with a focus on the environment, social and economic sensitivity, without leaving aside the balance of the ecological interactions of the production system; these systems have species biodiversity, with a tendency to resilience and to be energy efficient [1, 2]. Conventional management has production yields as a priority, and to achieve this based on a large percentage of industrial inputs such as improved seeds, agrochemicals and machinery [3].

Microorganisms are of vital importance with regard to the sustainability of an ecosystem, since they perform multiple functions and ecological services such as: the decomposition and mineralization of organic waste, the regulation of biogeochemical cycles, the contribution in the retention and release of nutrients in plants; generation, maintenance, renewal and soil fertility; atmospheric regulation of trace gases, regulation of animal and plant populations, among others [4].

Bacteria are microorganisms that have different functions in the ecosystems and the capacity to subsist in a great diversity of these, thanks to the fact that they adapt to extreme environments of temperature and pH, and are even able to survive in the presence of radioactive compounds [5, 6].

Another important feature of bacteria is their cell wall that has the ability to counteract osmotic pressure, and serves as a protective against physical, chemical and biological damage [7, 8], the metabolism of bacteria it is a peculiarity that helps them to survive in minimum conditions for growth, needing a source of carbon and nitrogen, water and various ions [9]. Due to all these characteristics, bacteria are related to a large number of ecological processes [10].

The processes related to the bioremediation of pollutants are of great relevance, since bacteria are a very important factor in this process, since they have the capacity to remove or degrade contaminants with high levels of toxicity; among these are a great variety of organic compounds [11, 12], pesticides [13] and heavy metals [14].

Pesticides when used indiscriminately can cause adverse effects such as poisoning and in addition to polluting the environment through their dispersion of wind, water and soil [15]. One of the most alarming effects in intensive production systems where large amounts of pesticides are used is the direct incidence in populations of native microorganisms of biological interest, decreasing their biofertilization effects and the biochemical benefits with which they help promote the growth of the plants [16, 17].

That is why attention should be paid to the use of pesticides, given that in agriculture, bacteria have various functions that are related to nitrogen fixation, with the

promotion of plant growth, biological control and bioremediation [18], also serve as inoculants in the composting of fibrous organic waste [19], have the ability to raise the pH of some soils and their metabolites can serve as fertilizers [20]. Some bacteria when colonizing the roots of crops promote growth and prevent the establishment of pathogens, as well as contributing to the absorption of water and nutrients [21].

Because certain bacteria are able to survive in adverse environments, for example in the presence of pesticides, it is necessary to consider the availability, mobility and toxicity of these compounds used in agriculture, as well as the physicochemical properties of soil, among other environmental factors. In the present work, we studied the metabolic activity of bacteria isolated from plots with different agricultural management in the presence of different substrates, their capacity for biofilm formation and inhibition or resistance in the presence of different pesticides. The objective of the present work was to evaluate the metabolic processes from bacterial soil isolates and evaluate their viability in the presence of Furadan, Esteron and Malation.

MATERIAL AND METHODS

Biological material

Bacterial isolates were obtained from soils located in Vicente Guerrero, Tlaxcala (agroecological management) with coordinates 19°25'24" N, 98°28'56" W, Los Mochis, belonging to the municipality of Ahome, Sinaloa (conventional management) with coordinates 25°84'06" N; 108° 97'97" W and Chachapa in the state of Puebla (conventional handling) with coordinates 19°04'26" N; 98°05'17" O.

In each plot, 10 zig-zag subsamples were taken to obtain a composite sample of 2 Kg of soil; the sampling was carried out according to NOM-021-SEMARNAT-2000, in which it is indicated that the sampling for agricultural soils should be 0-15 cm deep [22].

Bacterial isolation

It was resuspended in test tubes with 10 ml of nutritious broth 1g of each soil sample, incubated at room temperature and under agitation at 40 revolutions per minute (rpm) for 24 hours. Then, 10 µl were resected by stria on nutritive agar and incubated at 30°C for 24 hours. Subsequently, the selection and isolation of viable colonies was carried out.

Bacterial identification

A colony was taken from each of the isolated samples and they were grown in 2 ml of nutritious broth at 30°C for 24 hours, to subsequently place 1 ml of the culture in the identification medium Compact Dry "Nissui" EC, incubated at 30°C during 24 hours, with the identification readings interpreted according to the agar staining colors: the red-pink color indicates presence of coliforms, beige indicates *Pseudomonas* and blue indicates *E. coli*.

Metabolic activity assays

For each isolation, metabolic tests of organic matter assimilation were performed with three different carbon sources (starch (CAM1), sucrose (CAM 2) and dextrose (CAM3), protease activity, lipase activity and DNAse activity.

Assimilation of organic matter: The isolates were streaked in the medium CAM (Carbon Assimilation Medium) using as a source of charcoal dextrose, sucrose and starch, incubated at 30°C for 24 hours, and the reading was made by the presence or absence of growth.

Protease activity: casein agar was used, where each of the bacterial isolates was planted by means of an abundant central stria and incubated at 30°C for 24 hours. The reading of the test was carried out analyzing the appearance of a transparent halo around the bacterial growth.

Lipase activity: the bacterial isolates were streaked and incubated at 30°C for 7 days in the agar-glycerol medium, the presence of a precipitate around the bacterial growth is a positive test for glycerol hydrolysis due to the combination of $Ca2^{+}$ and the acids fatty acids released during your metabolism.

DNAse activity: the isolates were sown by stria and incubated at 30°C for 24 hours in the DNAse agar medium, and the test was revealed by adding 1 N HCl on the culture, the presence of a halo around the culture was considered a positive test.

Evaluation and quantification of biofilm formation

The isolates were cultured in nutritious broth for 24 hours at 30°C and the inoculum adjusted to $1X10^{6}$ CFU/ml. In 96-well microplates, 200 µl was poured into each well (with 4 replications), including the negative control, the microplates were incubated 24 hours at 30°C. After the incubation time, three washes were performed with sterile distilled water, then 250 µl of 1% violet glass was added for 5 minutes, in order to stain the bacteria present in the biofilm, the excess of dye was removed rinsing with distilled water, 250 µl of 99% ethanol was added and the optical density was read at 620 nm in a PoweamWHYM201 plate reader.

The cut-off point (DOC) was obtained by the average of the optical densities obtained in the negative control plus 3 standard deviations. It was considered as not producing biofilm those strains with an optical density (OD) lower than the DOC, weak producing strains to those with an OD higher than DOC and less than 2 DOC, moderate those that have an OD between 2 DOC and 4 DOC and strongly producers to those that have an OD greater than 4 DOC [23].

Viability tests in the presence of synthetic pesticides

The isolates were confronted with Furadan (Carbofuran at 33.2%), Esteron 47 (2, 4-D at 49.20%) and Malation 1000 (0.0-dimethyl phosphorodithioate of diethyl mercaptosuccinate at 83.60%), to determine its viability through the agar diffusion assay. 5 mm discs of sterile Whatman No. 1 paper were prepared and impregnated with solutions of each the pesticides in

concentrations of 10%, 50% and 80%. Each of the isolates was cultured for 24 hours at 30°C in nutrient broth to adjust the inocula to 1 10^6 CFU/ml. On Mueller-Hinton agar plates, a massive seeding of the cultures was carried out and 3 discs were placed equidistantly, 2 impregnated with one of the pesticides and one with Ciprofloxacin (positive control). The plates were incubated at 30°C for 24 hours; the test was interpreted by measuring the diameters of the halos formed around the discs.

Statistical analysis

The data obtained were managed with an analysis of variance and its respective multiple comparison of Tukey with a level of significance of 0.05 (Instant Software 2.0).

RESULTS

Bacterial identification

Strains isolated from the different study areas that were inoculated into the Compact Dry Nissui EC medium showed the following results: in the Los Mochis soils, isolates 4, 5 and 8 were coliforms and isolates 1, 2, 3, 6 and 7 *Pseudomonas*; as for the isolates from Vicente Guerrero, only isolation 5 corresponded to coliforms and isolates 1, 2, 3 and 4 to *Pseudomonas*; in Chachapa, isolation 1 was *Pseudomonas* and isolates 2, 3, 4 and 5 were coliforms.

Identification in Los Mochis, 62.5% of positivity to *Pseudomonas* and 37.5% to coliforms were presented; in Vicente Guerrero 80% to *Pseudomonas* and 20% to coliforms; and in Chachapa 20% to *Pseudomonas* and 80% coliforms.

Metabolic activity assays

Test of the metabolic activity the bacterial isolates developed better in the presence of dextrose and sucrose, the isolates of Los Mochis 4 and 8 presented greater growth in the different means of carbon assimilation. In the other tests, only one isolation was positive for each substrate, with isolation 1 positive for glycerol, isolation 6 for DNAse and isolation 5 for casein (Table-1).

In the Vicente Guerrero isolates, it was observed that the bacteria efficiently metabolize sucrose and dextrose, with isolates 4 and 5 growing more strongly (Table-2).

Regarding the Chachapa isolates, there was greater growth in carbon assimilation compared to the other two study areas, isolation 1 presented the lowest growth, however, it was positive for the DNAse test (Table-3).

Evaluation and quantification of biofilm formation

Of the isolations obtained from the samples from Los Mochis, 62.5% presented moderate biofilm production, 25% abundant production and 12.5% were identified as nonproductive. The area of Vicente Guerrero showed that 60% of the isolates do not produce biofilm, being weak and moderate producers with 20% respectively. With respect to Chachapa, 60% of isolates and 40% of non-producers were identified as weak biofilm producers (Table-4).

The tests on the isolates from Los Mochis showed a lower halo formation compared to the pesticides in the different concentrations compared to the Ciprofloxacin control, showing a significant difference ($p<0.05$).

Viability tests in the presence of pesticides

The results of the Vicente Guerrero isolates in the presence of Furadan showed a lower formation of haloes in the concentrations evaluated compared to the Ciprofloxacin control, showing a significant difference ($p<0.05$), likewise differences were found between the concentration haloes 10% with 50% and 80%. Esteron showed a significant difference ($p<0.05$) between the halos with the concentrations at 10% and 50% with respect to Ciprofloxacin.

Table-1. Metabolic activity in samples from Mochis, Sinaloa.

Sample	1	2	3	4	5	6	7	8
CAM 1	x	x	xx	xx	x	x	x	xx
CAM 2	xx	x	xx	xxx	xx	xx	x	xxx
CAM 3	xxx	xx	xx	xxx	xx	xxx	x	xxx
GLYCEROL	+	-	-	-	-	-	-	-
DNAse	-	-	-	-	-	+	-	-
CASEIN	-	-	-	-	+	-	-	-

X = Few growth, XX = Moderate growth and XXX = Abundant growth

Table-2. Metabolic activity in samples from Vicente Guerrero, Tlaxcala.

Sample	1	2	3	4	5
CAM 1	x	x	x	x	x
CAM 2	xx	x	x	xxx	xxx
CAM 3	xx	xx	xx	xx	xxx
GLYCEROL	-	-	-	-	-
DNAse	-	-	-	-	-
CASEIN	-	-	-	-	-

X = Few growth, XX = Moderate growth and XXX = Abundant growth

Table-3. Metabolic activity in samples from Chachapa, Puebla.

Sample	1	2	3	4	5
CAM 1	x	xx	xx	xx	xx
CAM 2	x	xxx	xxx	xxx	xxx
CAM 3	x	xxx	xxx	xxx	xxx
GLYCEROL	-	-	-	-	-
DNAse	+	-	-	-	-
CASEIN	-	-	-	-	-

X = Few growth, XX = Moderate growth and XXX = Abundant growth

Table-4. Biofilm formation determination.

Sample	Biofilm (Mochis, Sinaloa)	Biofilm (Vicente Guerrero, Tlaxcala)	Biofilm (Chachapa, Puebla)
1	Abundant	Moderate	Not producing
2	Moderate	Not producing	Weak
3	Moderate	Not producing	Weak
4	Abundant	Weak	Not producing
5	Moderate	Not producing	Weak
6	Moderate		
7	Not producing		
8	Moderate		

The Chachapa isolates showed a lower halo formation compared to the pesticides in the different concentrations compared to the Ciprofloxacin control for Furadan and Malation, showing a significant difference ($p<0.05$). Esteron showed a significant difference ($p<0.05$) when comparing the haloes between the concentrations of 10% and 50% with Ciprofloxacin.

In the presence of Furadan, the Vicente Guerrero isolates showed greater susceptibility, highlighting concentrations at 50% and 80%, presenting significant differences ($p<0.05$) with the Los Mochis isolates, since these isolates showed greater resistance to Furadan.

The isolates of Vicente Guerrero in the presence of Esteron at 80% presented the halos of greater diameter with $p<0.05$ compared to the isolates of Los Mochis and Chachapa, especially in the concentrations at 10%.

When comparing the isolates of Los Mochis v.s. Chachapa, no significant differences were found ($p>0.05$) in the diameters of the halos in the presence of the three pesticides. The Vicente Guerrero isolates showed significant differences ($p<0.05$) with respect to Los Mochis and Chachapa. Figure-1 shows the differences in the bacterial halos in isolated of Vicente Guerrero susceptible to pesticides and an isolated of Los Mochis that had a minimum inhibition against pesticides.

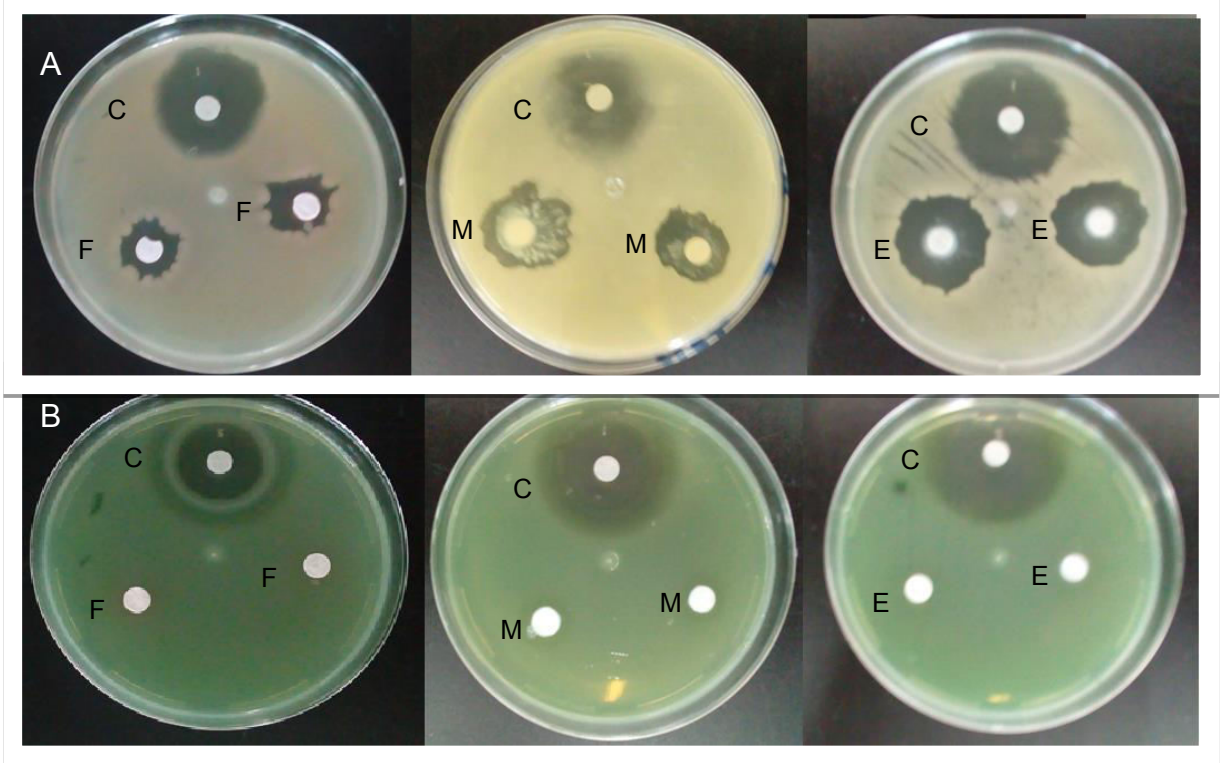

Figure-1. Viaibility test of bacterial isolates on Mueller-Hinton agar, in the presence of discs impregnated with 80% pesticides (C = Ciprofloxacin, F = Furadan, M = Malathion and E = Esteron). A: Vicente Guerrero isolated 2 and B: Los Mochis isolated 5.

DISCUSSIONS

The sampling plots of Los Mochis are totally oriented towards a conventional management where it is worth noting that a great diversity of agrochemicals is used, abundant use of machinery and use potatoes monoculture, squash, beans and corn predominates; the plots of Chachapa present a conventional management using corn monoculture, herbicides and machinery; and those of Vicente Guerrero have agroecological management making use in greater proportion of manual handling practices for the preparation of the field, polyculture predominates (corn, squash, beans) and crop rotation (maize with beans) and there is a minimum use of herbicides in some plots.

The bacterial identification in the total of the isolates of the soil samples had a higher percentage of the genus *Pseudomonas*, which have been reported in multiple works in the presence of different contaminants [24, 25]. It is likely that the genus *Pseudomonas* shows resistance to pesticides and can metabolize some of these compounds, however this capacity depends on several factors such as environmental conditions, physical characteristics, metabolic and time of contact with the pesticide [26].

The type of agricultural management of the aforementioned soil samples gives us a guideline on why isolates from plots with conventional management responded better to metabolic activity assays, especially in the activity of proteases, lipases and DNAse, and the isolations of the samples of plots tending to an agroecological management were negative to these tests. This can be due to the fact that the use of pesticides decreases soil microbial diversity and modifies its metabolic activity, an example is the decrease of nitrification and oxidation of methane, because nitrification is very sensitive to stress by contaminants, being inhibited by pesticides [27-30].

Regarding the formation of biofilm, this was higher in the isolates of Los Mochis, Sinaloa, followed by the Chachapa isolates, with the Vicente Guerrero samples being the group with the lowest biofilm formation. The fact that the Los Mochis isolates have shown greater biofilm formation could be due to the fact that the bacteria adapted to the conditions of agricultural management, especially to the indiscriminate application of agrochemicals, because the insecticides and herbicides despite not having the In order to fight the bacteria, they can have an inhibitory effect on these causing alterations in the formation of biomass and its metabolic activity [31]. Given this scenario, biofilm-forming bacteria can be more abundant and successful since they are in a place with adverse conditions, with bacteria that adapt to the presence of substances with a biocidal effect surviving in a greater proportion [32].

The biofilms favor the survival and adaptability of the bacteria, because they are composed of extracellular polymeric substances that surround them, likewise these substances give the bacteria the ability to adhere to different structures, protecting them against antimicrobial

substances and toxins, maintaining the hydration for a longer time facilitating the nutrients obtaining, reducing environmental adversities, among others [33-36].

According to the above, it can be assumed that the Los Mochis isolates showed greater viability thanks to the formation of the biofilm, being able to exchange genetic material despite being of different species, facilitating the generation of the necessary plasticity based on the adverse conditions of their environment [37]. Due to the characteristics of a biofilm, these are being used in bioremediation techniques of xenobiotic contaminants such as pesticides, and the ability to adhere helps them to bioremediate gritty sites [38].

Regarding the relationship between bacterial identification and biofilm formation, there was no variation, since biofilm formation occurred in the *Pseudomonas* and coliform isolates.

In the tests in the presence of pesticides the averages of the different percentages by sampling area showed that the herbicide Esteron promoted greater bacterial inhibition, later the Malation, and the Furadan had less inhibition in the isolations.

Benitez *et al*., (2009) conducted experiments with different pesticides in which the herbicides showed a mildly toxic effect above the insecticides and below the fungicides, which represent the highest toxicity for bacteria (*Bacillus subtillis*). There was also a clear difference between Furadan and Tordon 101 herbicide, describing Furadan as a pesticide that does not have a significant toxicity in bacteria, on the contrary Tordon 101, which contains among its compounds 2, 4 D was the most effective herbicide toxic reported. This clarifies why the herbicide Esteron (2.4 D) showed the highest bacterial inhibition as opposed to Malation and Furadan insecticides and acaricides [39].

Despite the fact that 2, 4-D can cause bacterial inhibition, some bacteria, such as *Rhizobium*, despite having adverse effects with this pesticide, may at some point change their metabolism in order to assimilate[40].

The Malation was the pesticide that induces larger diameter halos after Esteron, and in contrast to Furadan has been found in different works that can inhibit microorganisms, an example is a study where the level of inhibition of microorganisms in the rice culture, in which it was affirmed that Malation has a significant reduction on phosphorus solubilizing microorganisms [41]. Malation has been reported to decrease the abundance of microbial diversity in activated sludge at concentrations of 0.1 mg / l and 3 mg / l at 40-day exposures [42].

In the experiments it was observed that the most susceptible isolates to the pesticides were those of Vicente Guerrero, excelling Malation and Esteron in all their concentrations for the bacterial inhibition that they present, this can be due to the type of handling in which there is minimum use of pesticides. In contrast, the isolates of Los Mochis and Chachapa showed less inhibition in the presence of pesticides, which can be explained according to some glyphosate studies, which showed that there is less microbial vulnerability to the pesticide after a new application, that is, bacteria are less sensitive to pesticides in soils with a previous history of pesticide application [43].

Similarly, another study showed that a soil with no history of glyphosate application proved to be more sensitive than one with previous application [31].

It is important to manage the appropriate concentrations of pesticides and discontinue those that are internationally and nationally prohibited, since many pesticides modify the microbial environment and therefore the biological processes will be modified, a clear example is the decrease of the microbiological diversity and the increase of strains that are in interaction with pesticides [44, 45], such as Metal-sodium that is described in the work of Li *et al*., (2017) where it was observed that in presence of this pesticide there was an increase of the genera *Paenibacillus* and *Luteimonas*.

Regarding the relationship between microbial diversity and the biological processes of soil due to the effect of pesticides, some studies claim that this interaction is not modified by decreasing the diversity of microorganisms, conserving the mineralization of carbon and nitrogen at normal levels [46]. On the contrary Griffiths *et al*., (2000) affirm that the nitrogen cycle in the soil is affected by the decrease of bacterial diversity, in the same way the oxidation of methane is affected [28].

CONCLUSIONS

Organisms isolated from plots with conventional management had greater activity in biochemical tests and biofilm formation. Regarding viability in the presence of pesticides, these bacteria showed a lower inhibition of their growth. The isolates of Los Mochis and Chachapa had the highest metabolic activity and biofilm formation, with the Vicente Guerrero isolates showing the lowest metabolic activity and biofilm formation.

The isolates from Vicente Guerrero presented the greatest inhibition against Furadan, Malation and Esteron, and the isolates from Los Mochis had the least inhibition.

The pesticide that showed the highest bacterial inhibition was Esteron, followed by Malation and Furadan, the latter having the least inhibition in the isolates and concentrations evaluated.

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