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Isolation of entomopathogenic fungi from soils of the Comarca Lagunera as a biological control of pests

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Abstract

The main objective of this study was to isolate entomopathogenic fungi with the ability to degrade exoskeleton chitin from insect pests as the only source of carbon. The fungi isolated in soils from La Comarca Lagunera were inoculated in minimum salt medium (MMS) supplemented with shrimp cuticle (1%). From 14 strains isolated, only 8 showed entomopathogenic activity, presuming the presence of genera *Beauveria* and *Metarhizium*, identified by microscopy with lactophenol blue. In order to corroborate the enzymatic and entomapatogenic activity, the isolates were cultivated in MMS in the presence of domestic pests from the northern region of this country (*Acheta domesticus*, *Blatella germanica* and Musca domestica) showing activity at 72 h^{-1} and having their best yields at pH 4. The results obtained in the increment of biomass with the strains AG4 and VE (yields of 70 and 52% respectively) in colloidal chitin at 72 h^{-1} may be the precursor of strategies for biological control of pests in this region.

Entomopathogenic Fungi, Biological Control, Pests, Chitin

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1. Introduction

The use of pesticides and insecticides for the control of pests, in favor of the protection of crops, had been the commonly used and useful strategy, but it has also caused damage to health and the environment. There are around 15,000 species that are considered pests, of which 300 species require a timely form of control (Tarek Mohamed, 2002).

The indiscriminate use of pesticides has caused that many insects and mites have become pests much more difficult to control before the use of these synthetic insecticides. This has caused a serious problem in sectors such as horticulture, forestry and agriculture. The environmental and social costs associated with the use of pesticides, reaches about 8 billion dollars each year (Osteen et al., 1993). In a study conducted by the WHO (1990) (cited by Eddleston et al., 2002) between 500,000 and 1 million people are poisoned with chemical pesticides annually and approximately 5,000 to 20,000 lose their lives.

At present, a strategy that is being implemented for the indiscriminate use of pesticides is the application of biological agents; for example, viruses, bacteria, protozoa and fungi. A viable alternative for this problem is the use of entomopathogenic fungi. These microbial agents have a great potential as controlling microorganisms, constituting a group with more than 750 species disseminated in the environment (Díaz et al., 2006). Among the main species of entomopathogenic fungi are Beauveria bassiana, Metarhizium anisopliae, Isaria fumosorosea, Paecilomyces spp, Verticillium lecanii, Smittium morbosum, among others. The majority of these fungi belong to the genera of Chytridiomycetes, Deuteromycetes, Ascomycetes, Zygomycetes, and Oomycetes, found to a large extent in nature (Gul et al., 2014).

For several years, these fungi have been shown to be an important technique for effective management in the biological control of pests. The mechanism of action of entomopathogenic fungi is given by a balanced interaction between the fungus, the host and the environment. The life cycle includes an infectious sporulation stage that germinates in the host's cuticle, forming a germ tube that penetrates the cuticle and invades the insect. The potential increase of infectious spores generates the production of toxins that end the life of the insect (Hajek and St. Leger, 1994).

In this context, it is important to generate new alternatives that favor the indiscriminate use of pesticides and insecticides. Entomopathogenic fungi are a good strategy since there is a wide variety of these microorganisms, they can be easily cultivable in the laboratory and can survive in a parasitic or saprophytic manner in the host.

The objective of the present work was to isolate and cultivate different soil fungi to carry out bioassays with shrimp cuticle, Acheta domesticus, Blattella germanica and Musca domestica with the purpose of analyzing their quitinolitic capacity and alternative biological control to the use of pesticides and insecticides.

1.1 Justification

Currently, it is essential to avoid further deterioration and pollution to the environment. In the area of agriculture, few ecological alternatives have been implemented to help this problem. One of the main strategies is the control of pests through the use of biological agents. The implementation of entomopathogenic fungi in agriculture potentially helps to reduce the adverse effects produced by the indiscriminate use of pesticides, both for the environment and for the human being.

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The carrying out of integrated pest management is of vital importance for insects to be combated by means of natural enemies that can: prevent damage to the ecosystem, decrease the economic resources allocated for that purpose, and not alter the chemical composition of the food, develop a sustainable agriculture and not cause harm to the health of people.

In addition to the above, the cost-benefit can be obtained in the short term, because it can reduce the use of conventional chemicals without minimizing the production of the harvest.

1.2 Problem

At present, the issue of environmental pollution is becoming stronger. Population growth has caused an excessive demand for food, including fruits and vegetables. To supply these food needs, the use of pesticides is the main alternative for the protection of crops, these substances have been the strategy commonly used, but also, they have caused negative impacts to the environment, due to excessive and indiscriminate use.

Pesticides have been shown to be related to health problems in humans, the residence time they can have in soil and water is considerable, they can bioaccumulate or biotransform into compounds more toxic to humans and the environment.

Likewise, the inappropriate use and excessive application of these chemical substances brings about the deterioration of the environment caused by the direct application to the crops, their incorrect storage and accidental spills, so that, these substances are dispersed in the environment affecting the the biotic and abiotic factors of the ecosystem.

So it is convenient to find new strategies to solve these environmental and public health problems. One of the most viable are entomopathogenic fungi; which, they stand out for having the metabolic ability to infect insects and cause their death.

The use of biopesticides based on this type of microorganisms helps to reduce the environmental impact coming from the pesticides, they are selective and show a high specificity. Likewise, they have the ability to multiply and spread their spores in the environment, producing an important defense mechanism to the pest. These characteristics make the entomopathogenic fungi a viable strategy for the biological control of pests.

1.3 Hypotesis

– Research hypothesis: Fungi isolated from different sites of the Comarca Lagunera have the capacity to degrade insect pests in the region.

– Alternative hypothesis: Fungi will show better growth and greater chitinolytic ability at acid pH.

– Null hypothesis: Expression of extracellular proteins in MMS media enriched with colloidal chitin will not be proportional to the amount of reducing sugars.

1.4 Objectives

1.4.1 General objective

Analyze the degradative potential in insects that have entomopathogenic strains isolated from different soils of the Comarca lagunera as an alternative to the biological control of pests.

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1.4.2 Specific objectives

- Isolate entomopathogenic fungi from humid soils of the region of the Comarca Lagunera.
- To determine the ability of isolated fungi to hydrolyze chitin by bioassays with shrimp cuticle and biomass generation.
- Analyze the chitinolytic activity by a colorimetric technique.
- To evaluate the pathogenicity of the strains implementing bioassays with insects considered pests in the region.

2. Theoretical framework

Biopesticides based on different microorganisms are considered good control agents for harmful insects for agricultural crops. For example, the use of entomopathogenic fungi in the biological control of pests has increased globally in recent decades, yielding good results (Faria and Wraight, 2007).

There are two ways in which an affectation by the fungus to the insect can happen, one is the chitinolytic activity that the fungus has and the second is through the production of toxins. The mechanism of pathogenicity of both processes of these fungi has been studied in such a way that currently the main stages of the infective process are already known; Thanks to this, strategies have been established to improve its application as biological control.

Entomopathogenic fungi unlike other entomopathogenic agents have unique mechanisms of action since they do not need to be ingested by the insect to control it, but it infects it by contact and adhesion of the spores to different parts of the body of the insect, causing vital damage to the pathogen (Ortiz-Urquiza and Keyhani, 2003).

From a general perspective fungi present the following development phases as mentioned by Alean Carreño (2003), the mechanism of action is divided into three phases: The first phase consists of the adhesion and germination of the spore to the surface of the exoskeleton of the insect. In this process there is an interaction between the hydrophobins found in the conidium and the hydrophobic surface of the exoskeleton. Subsequently, penetration takes place in the second, a process in which, according to the species of fungus, a mucilaginous substance is formed that holds it together to form a germinal tubule that penetrates through the pores and outer layers of the epicuticle. Finally, the last step is the growth of the fungus. Which usually results in the death of the insect.

The death of the insect occurs more quickly when it is affected by a fungus that produces considerable amounts of toxins. Among these toxins are the destruxinas (demetildestruxina and protodestruxina). Its mode of action is to inhibit the synthesis of DNA, RNA and proteins in the cells of insects (Pucheta et al., 2006); in addition, they are substances of low toxicity, but of much virulent activity on insects, mites and nematodes.

Everything described above favors the organic composition of the exoskeleton of insects. The cuticle of the insects consists of crystalline chitin nanofibers, some of which are deacetylated and play an important role, since they allow the formation of resistant and support tissues for arthropods, arachnids, insects, nematodes, annelids, brachiopods, mollusks, among others. Also this carbohydrate is part of the cell walls of some fungi; as ascomycetes, basidiomycetes, which are also composed of glucans and other polymers (Ezekiel et al, 2014). Chitinase is an enzyme that can hydrolyze chitin and has been found not only in fungi, but also in bacteria, plants, vertebrates and invertebrates.

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They have an important role in the process of molting of insects, the digestion of chitinous foods and as a defensive enzyme against chitinous pathogens. Entomopathogenic fungi synthesize metabolites that are toxic against insects and, generally, are synthesized when the fungus has penetrated the exoskeleton (Téllez-Jurado et al., 2009).

The entomopathogenic fungi provide excellent solutions for biological control. The panorama is very promising and this can be extended to find uses as in the field of agriculture and, in this way, to develop an integrated management of insects considered pests and fungi as part of a sustainability that contributes to reduce the effects of environmental pollution. The deciphering adequately the mechanism of action of enzymes and toxins, will allow an important step to the development, generation and implementation of bioinsecticides, developing research in the field of biotechnology, agricultural and environmental.

3. Materials and methods

3.1 Sampling

A total of 6 samples were collected from humid soil from different points of the urban area of the Comarca Lagunera under aseptic conditions. Each sample contained approximately 10 g of soil deposited in sterile polyethylene bags. These samples were stored at 4 ° C for later use (Feng and Yang, 2010).

3.2 Isolation and cultivation of fungi

The samples were isolated by the technique of serial solutions using as medium Papa Dextrose Agar (PDA); to which 1% gentamicin was added to prevent the growth of bacteria. After 72 hours of incubation at 27 ± 2 ° C, a significant growth of fungal colonies was observed. The individual colonies were sub-cultivated and stored on agar inclined with PDA and antibiotic at 4 ° C for their conservation.

This procedure was repeated three times in order to have isolated colonies with entomopathogenic fungal characteristics (García et al., 2011).

3.3 Morphological characterization

To morphologically characterize the isolated fungi were stained with the reagent Lactophenol Cotton Blue (LPCB) and were observed under a microscope at 100X (Hamiduzzaman et al., 2012). For the macroscopic and microscopic characterization of the isolated species, the criteria established in their research by Mier et al. (2002) were taken into account, including colony color, consistency, surface area, growth rate, appearance, size and hyphae.

3.4 Bioassays with shrimp cuticle

The isolated fungi were planted in a minimal medium of salts with colloidal chitin as the sole carbon source. First, colloidal chitin was prepared using dry shrimp. The shrimp was washed with distilled water and placed in 2N HCl for 24 hours. Afterwards, it was washed, dried to detach the cuticle and macerated to obtain a fine powder that passed through a 40 mesh screen. It was stored at 4 ° C until its later use (Castro et al., 2011).

Then, a minimum salt medium (MMS) was prepared (2.0 g of KH_2PO_4, 0.4 g of $CaCl_2 \cdot H_2O$, 0.3 g of $MgSO_4 \cdot 7H_2O$, 37 mg of $CoCl_2 \cdot 6H_2O$, 50 mg of $FeSO_4 \cdot 7H_2O$, 16 mg of $MnSO_4 \cdot H_2O$, 14 mg of $ZnSO_4 \cdot 7H_2O$ for 1 L) (Jackson et al., 1997) with 1% of Gentamicin, enriched with a concentration of 1% colloidal chitin. Treatments were carried out with pH 4, 5 and 7 with a final volume of 50 mL. The pH was adjusted with H_3PO_4 and NaOH. Finally, the medium was distributed in glass jars in which the previously obtained strains were inoculated in triplicate. They were incubated at 27 ± 2 ° C for a minimum of 72 hours or until mycelial growth was observed.

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3.5 Determination of biomass

To analyze the biomass growth of the fungi, the dry weight was determined according to the methodology proposed by García-Gutiérrez et al. (2013). First, fungi were allowed to grow for 72 hours in MMS with shrimp cuticle. Subsequently, aliquots of 5 mL were taken to determine the biomass produced by the dry weight technique.

The samples were filtered through a WhatmanTM No. 42 filter paper, previously dried at 75 ° C for 24 hours. Subsequently, they were cooled in a desiccator at room temperature and the filters were weighed, obtaining the grams of the fungus. This process was carried out in an initial and final stage of the research process at pH 4, 5 and 7 in triplicate.

3.6 Determination of chitinolytic activity

To determine microorganisms with positive chitinolytic capacity, a basal medium containing 0.3 g of MgSO4 • 7H2O, 3.0 g of (NH4) 2SO4, 2.0 g of KH2PO4, 1.0 g of citric acid monohydrate, 15 g of agar, 200 g was prepared. µl of Tween-80, 4.5 g of colloidal chitin and 0.15 g of bromocresol purpura (for 1 L of final volume).

The pH was adjusted to 4.7 and autoclaved at 121 ° C for 15 minutes. The medium was emptied into Petri dishes for polymerization. To check the chitinolytic activity, the isolated strains were inoculated with digralsky loop in the medium. They were incubated at 25 ± 2 ° C for 3 days to see the formation of colored zones. Trials were conducted in triplicate (Agrawal and Kotasthane, 2012).

3.7 Determination of reducing sugars and crude protein

To determine reducing sugars, a calibration curve was made with different concentrations of dextrose (100-1000 mg / L), these were reacted in a 1: 1 ratio with 3,5-Dinitrosalicylic acid (DNS), then heated to a bath Maria for 10 minutes and measured at a wavelength of 545 nm (Miller, 1959). The Bradford (1976) trial was used to measure the amount of crude protein intra and extracellular. A standard curve was made with albumin (100-500 mg / L).

The reaction was performed with the following ratio 1: 7: 2 (sample: dH2O: Bradford reagent) and measured at a wavelength of 595 nm. The determination of reducing sugars was evaluated at the beginning and end of the process, the protein was evaluated every 24 h-1.

3.8 Bioassays with insects

To check the entomopathogenic capacity of the fungi, some modifications were made to the protocol proposed by Gutiérrez and collaborators in 2014. The bioassays were elaborated with MMS and insects that are considered common pests in the region. Insects were obtained such as Acheta domesticus, Blattella germanica, and Musca domestica; which were previously washed with a 5% sodium hypochlorite solution to disinfect them and, before the medium solidified, they were added to the medium. 100 µL of the fungus adjusted to 0.5 was inoculated on the McFarland scale. Plates were incubated at room temperature until mycelial growth was observed.

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4. Results and discussion

4.1 Isolation and cultivation of fungi

Of the 6 sites sampled, a total of 14 isolated strains were obtained; of which, only 8 were those that showed ability to adapt better to the minimum salt medium with shrimp cuticle. The strains (AM, VE, NE, AG1, AG2 and AG4) had mycelial growth after 72 hours of incubation. On the other hand, strains AG3 and AG5 showed a slow adaptation to MMS and a mycelial growth was observed after 96 hours. The experiment was carried out in triplicate and the strains were reseeded at least 3 times until macroscopic typologies characteristic of the fungus were obtained.

4.2 Morphological characterization

The morphological characterization was performed by the differential technique of lactophenol blue. The microscopic features of hyphae and vegetative mycelia were determined to identify genera of fungi with entomopathogenic properties (Beveridge et al., 2007).

The isolated strain AG1 was identified by the genus Beauveria. This strain was characterized by a slow and circular growth of white color for 10 days at room temperature. The colonies were approximately 20 mm in diameter, their appearance was woolly and at first the colonies turned yellow. Likewise, it presented a soft texture with a flat surface, similar to what is established by Domsch et al. (1993).

In section A of figure 1 the strain AG2 is shown, which shows the morphological characteristics of the genus Metarhizium. It was observed under the microscope that it has cenocitic hyphae and the olive green color was well defined in the culture. Its growth was a little faster reaching 38 mm in diameter in 10 days at room temperature.

The fungal colonies grew in a circular manner with cottony appearance, presented a flat surface and a soft texture (Domsch et al., 1993, Mier et al., 2002). With respect to the other isolated fungi, it is even more pretentious to ensure the identity of their genus by its macroscopic and microscopic morphological.

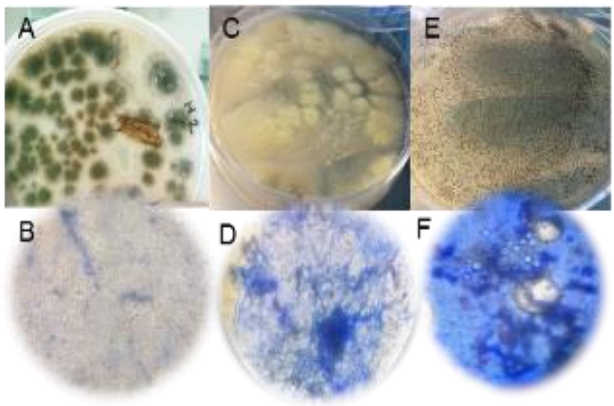

Figure 1 Isolated strains of entomopathogenic fungi from moist soils. A) Cultivation fungus Metarhizium, B) microscopy at 100 X of the fungus Metarhizium, C) Culture fungus Beauveria, D) microscopy at 100 X of the fungus Beauveria, E) Culture of the isolated strain AG5 and, F) microscopy at 100 X of the fungus AG5.

4.3 Bioassays with shrimp cuticle

75% of the strains showed a mycelial growth close to 72 hours after being inoculated in MMS with colloidal chitin as the sole carbon source. The rest of the fungi obtained a visible macroscopic growth after four days of incubation at room temperature. Being NE, AM and AG5 those that presented these characteristics. The size of the strains ranged between 10-40 mm in diameter (Figure 2).

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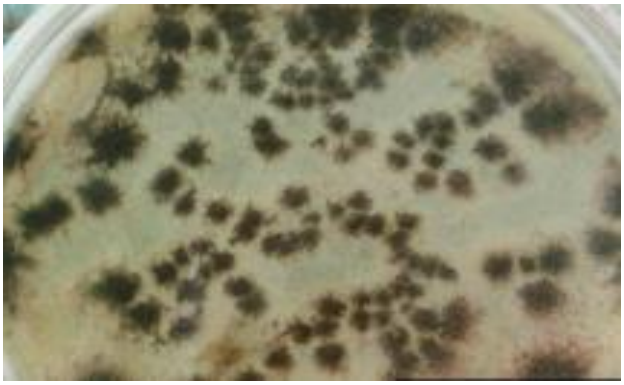

Figure 2 Growth of isolated NE fungus in MMS with colloidal chitin as sole carbon source

There is evidence that fungi that grow under chitin conditions as the only carbon source and with the presence of ammonium salts as an inorganic source of nitrogen, generate metabolites, including enzymes, that favor their reproduction and hydrolysis of β1-4 bonds. of the N-acetylglucosamine units (Shanmugaiah et al., 2008).

4.4 Determination of biomass

The biomass production was measured after 10 days of growth. This analysis allows to identify the strains that showed the highest growth in the MMS medium with colloidal chitin as the sole carbon source (table 1). The AG4 strain inoculated at pH 4 obtained the highest yield (70%) at pH 4 and 25% at pH 5 and 7. Likewise, the fungus referenced as VE increased its biomass by 52% at pH 7 and 24% at pH 5. On the other hand, strains NE (pH 5 and 7), AG3 (pH 4 and 5), and AG5 (pH 4, 5 and 7), had a lower yield in biomass production; which, grew below 20%.

The growth in biomass of the fungi is related to the ability to capture the organic nitrogen present in the environment. Nitrogen favors hyphal reproduction (Eyal et al., 1994) and this can proceed by hydrolyzing the chitin present in the insect as a source of carbon and nitrogen.

4.4 Determination of chitinolytic activity

The basal chitinase detection medium was supplemented directly with colloidal chitin (4.5 g / l) and bromocresol purple dye (0.15 g / l) (Agrawal and Kotasthane, 2012). The result was a bright yellow color. When chitinolitic activity occurs, the medium turns to colorations between purple and pink.

In figure 3 some of the strains that showed good chitinolytic activity are shown. The color turn began at approximately 72 hours of incubation at room temperature. Isolated strains AM, NE and VE showed the highest chitinolytic activity after 10 days of incubation, compared to the rest. What is significant is a slow adaptation to the basal medium with colloidal chitin as a carbon source, because in the beginning the degradation of chitin is complex and slow.

pH	Strain	% biomasa
4	AM	43
7	AM	45
4	NE	39
5	VE	24
7	VE	52
5	AG1	28
7	AG1	25
4	AG2	32
5	AG2	28
7	AG2	29
7	AG3	32
4	AG4	70
5	AG4	25
7	AG4	25

Table 1 Percentages of most significant biomass yields of isolated fungi

This is indicative that fungi excrete some enzymes amylases, proteases and chitinases that are related to the pathogenicity of the fungus, specifically in the part of adhesion and germination of spores (Chernin et al., 1997).

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Other research indicates that the chitinolytic activity is directly related to the enzymatic expression of the fungus to the extracellular medium. This would allow to degrade the exoskeleton of the insect by releasing an enzymatic complex that includes hydrolytic enzymes, among which stand out, the chitinases (Michel et al., 2005).

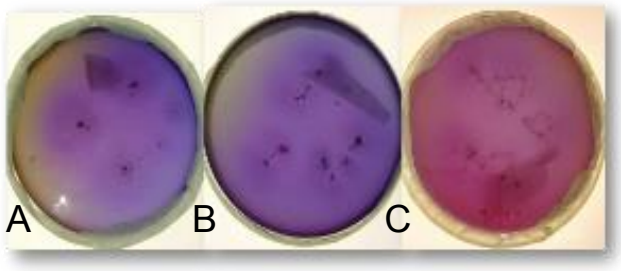

Figure 3 Chitinolytic activity of strains isolated in a medium supplemented with colloidal chitin after 72 hours. a) AM, b) NE and c) VE

4.5 Determination of crude protein and reducing sugars

Chitin is a potent inducer in the production of chitinolytic enzymes. When this carbohydrate is present in the medium as the sole source of carbon, the fungus triggers a series of metabolic processes for its growth. The concentration of reducing sugars, intra- and extracellular proteins was calculated, both at the beginning and at the end of the experimental process, to relate it to growth.

A Pearson correlation analysis with a significance level of .05 was carried out. The correlation between the extracellular proteins expressed by the fungus and the amount of reducing sugars showed a negative relationship between the variables. As more reducing sugars are expressed, the extracellular proteins may have a partial decrease in the medium.

It can be inferred that due to the low amount of N-acetylglucosamine, due to enzymatic hydrolysis, some quito-oligosaccharides were formed, such as chitobiose and chitotriosa, which could not be degraded by the fungus. In contrast, statistically the relationship between intracellular proteins was directly proportional to the final biomass concentration of the fungus. This may be an indication that proteins are enzymes that can be exported to the extracellular medium to degrade chitin. On the other hand, the data obtained from the growth of microorganisms at different pH were subjected to the analysis of variance (ANOVA), with a reliability coefficient of 95% ($p = 0.05$) to determine which treatment has greater efficiency in the production of proteins. Extracellular Statistically, isolated strains show better enzymatic activity at a pH of 4.

The above is related to biomass, since the fungi AG2, AG4, NE and AM generated higher biomass yield at pH 4. Some research shows that genera of Beauveria and other entomopathogenic fungi can grow in conditions where there are few nutrients and in a wide range of pH (Shimazu and Sato 1996).

4.6 Bioassays with insects

For the final bioassays, tests were carried out with Acheta domesticus, Germanic Blattella and Musca domestica; which, can be considered as plagues in the region. In the first place, the Acheta domesticus species (Figure 4, section A) was vulnerable to all the isolated strains. Growth was shown after 96 hours of incubation at room temperature. The fungi grew irregularly by the trunk of the insect and the Petri box. In the case of the Blatella germanica species (Figure 4, section B), a greater development of the AG1 fungus (genus Beauveria) was observed in the insect compared to the other isolates. The fungus showed a mycelial growth on the insect starting at 72 hours, covering part of the trunk and extremities.

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It is worth mentioning that it was the insect that had the greatest resistance to the growth of microorganisms. Only considerable growth was observed with strains AG1, AG4 and AG5. On the other hand, a significant affectation was observed in the species Musca domestica (Figure 4, section C) by the fungus AG5 that attacked a large part of the trunk and wings. Said growth was visible after 72 hours of incubation.

Tests were carried out on each of the 8 species of fungi that adapted to the minimum environment, mainly focused on the species Acheta domesticus, where a large general affectation was observed on the trunk of the species by the strains AG1, AG2 and AG5.

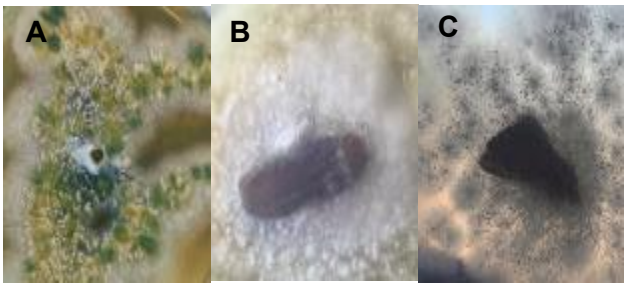

Figure 4 Bioassays performed with different insects considered as pests. A) Mushroom growth of the genus Metarhizium on Acheta domesticus at 96 h-1. B) Growth of the fungus of the genus Metarhizium on Germanic Blatella and C) Growth of the fungus AG5 on the moth

Previous studies have shown that entomopathogenic fungi, including Beauveria and Metarhizium species, produce proteases, chitinases and lipases that can degrade the cuticle of insects, favoring colonization (Charnley, 2003). The pathogenesis of the fungus on insects implies several factors for it to be carried out. The main factors are the host, the parasite and the environment; as well as, the metabolic capacity of the fungus so that it can degrade chitin. The penetration of the fungal pathogen in the cuticle depends in a relevant way on the action of the enzymatic activities (Castellanos-Moguel et al., 2008).

In this study the strains that showed the most activity on the pests were those that grew in the exoskeleton of the insect after 96 hours.

4.7 Future perspectives

There are several points that should be considered in further investigations such as: a) the selection of isolates with high chitinolitic potential for the infection of pests and the use of their biological derivatives (enzymes), b) the correct characterization of the most important fungal genera in this area of study, c) the study of the activity on pests present in crops of the region and d) the planning and implementation of technologies that can use this phenomenon of in vivo pathogenicity.

5. Conclusions

Of the 14 asylums, only 8 showed the ability to use chitin as the sole source of carbon and at the same time entomopathogenic activity on the genera Acheta domesticus, Germanic Blatella and Musca domestica, the former having the most exoskeletal involvement. There is a proportional relationship between the chitinolytic activity and the concentration of reducing sugars, presuming the presence of strong enzymatic mechanisms in strains AM, NE and VE, which served as the basis for determining the chitinolytic activity by means of the colorimetric test, taking its best yields at pH 4.

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