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Comparison in vitro of the invasive capacity of two formulations based on beauveria bassiana as a biological control of opsiphanes cassina.

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***Abstract*—**Opsiphanes cassina is the most common defoliating pest in the country's commercial oil palm plantations, being responsible for low yields and the disappearance of large numbers of hectares of cultivation. The objective of this work was to evaluate in laboratory the invasive capacity of two formulations (powder and liquid presentation) based on Beauveria bassiana on the insect Opsiphanes cassina. Microbiological quality control tests showed germination percentages of B. bassiana spores for the solid formulation of 90% while in the liquid they were 97.7%. 100% purity was achieved for both formulations and the concentration of spores was 4.9x1010 spores/ml in the solid formulation and 8.6x1010 spores/ml in the liquid formulation. Concluding that the liquid formulation causes greater mortality in a smaller time (6 days) on the larvae of the III instar, being feasible its production and commercialization.

Keywords: Beauveria bassiana, biological control, opsiphanes cassina, palm of oil, invasive capacity.

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I. INTRODUCTION

The oil palm is of great interest in agriculture and agroindustry due to its participation in the production of oils and its use to produce primarily food and products for human consumption. It is a plant adapted to tropical conditions [1], and due to its condition of introduced and permanent cultivation it allows the stabilization of the new agroecosystem through an adequate management of the plants as the central axis of the productive system and of the ecological environment; the quality of this management depends to a great extent on the levels of incidence of economically important native pest insects.

The oil palm cultivation is of great importance for Colombia since it is one of the main producers of this economic item, however, by 2013 the governorate of Norte de Santander, Colombia, reported for the municipality of Tibu 13.404 hectares of area planted with palm oil of which presented a yield for this year of 2.9 tons per hectare of palm with a total annual production of 38,842 tons and a loss of 10 hectares of crop represented by diseases that attack it, where the defoliation of the palm stands out. This disease caused by Opsiphanes cassina, affects almost all palm areas in the country, in most cases is responsible for low yields, because it causes severe defoliations in the palms, thus causing the disappearance of large numbers of hectares of crop.

The management of this pest can be done by capturing adults, significantly reducing its population through the use of baits prepared with ripe fruit chopped which are impregnated with some insecticide, although the indiscriminate use of these baits can be negative for natural enemies [2]. On the other hand, the use of chemical products has some disadvantages, such as the toxicity that affects the health not only of those in charge of fumigation but also of those who consume agricultural products, since they contain materials that are foreign to the environment and consequently can cause contamination and pollution to it [3].

For this reason, biological control is considered as an ecologically important and economically viable strategy, due to its contribution to the reduction of the use of highly toxic insecticide products in the environment [4]. Among microbial pesticides are entomopathogenic fungi considered the most effective alternative method with great potential as controlling agents, one of the most important kinds is Beauveria bassiana, this fungus affects arthropods, penetrating their cuticle, producing disease and insect's death. The formulations consist of combinations, so that the conidia remain stable and effective. Their colonies in agar potato dextrose at 14 days are cottony to powdery and white, and as time passes by, they become yellowish, creamy. And on the reverse, they are reddish in the center and yellowish around [5]. It has the advantage of being a biological pesticide that does not affect beneficial organisms and does not cause pollution to the environment. Its action is by contact and its conidia act in the different stages of the insect plague, making the insect sick, which led it to stop feeding and then die [6].

In this investigation two formulations were used, one solid and one liquid based on B. bassiana, and are described the bioassays carried out in the laboratory to determine the invasive capacity of B. bassiana on the III instar larva of O. cassina defoliator of the oil palm.

II. METHODOLOGY

a. Biological material

For the trials, larvae from the III instar of O. cassina collected at the COOPAR plant in the municipality of Zulia, Norte de Santander, were used, where outbreaks of oil palm defoliation disease and a B. bassiana strain from an original culture donated by the Universidad Francisco de Paula Santander´s collection were detected.

b. Isolation of Beauveria bassiana

The isolation of B. bassiana was performed on PDA (MERCK) with 1µl Ampicillin and incubated for 8 days at room temperature. Yeast extract (yeast extract 5g/L plus glucose 10g/L) was used for the preinoculum.

c. Massive production of the fungus

For the solid formulation, glass bottles with 80 g of rice plus 80 ml of distilled water were used and sterilized at 121°C with 15 lb for 15 minutes. Then 8 ml of the pre inoculum was inoculated into each glass bottle and left for 20 days in incubation at 25°C ± 2 until uniform growth was achieved. For the liquid formulation, sterile yeast extract broth was used, the fungus B. bassiana was inoculated and left for 8 to 15 days at room temperature until a concentration of approximately 1x1010 conidia/ml was reached.

Afterwards, the product was dried at a temperature no higher than 28 °C, with relative humidity (<70%), without the entrance of solar rays. The material was kept for 15 days, until the moisture content dropped to 12 or 14%, according to recommended protocol by Gomez and Mendoza [7] and was stored in refrigeration (4 to 8°C) until its use in the bioassays. In the case of the liquid formulation, the liquid matrix was used, and to stop the sporulation process it was cooled (4 to 8°C) until it was used in the bioassays.

d. Microbiological testing of formulations

Microbiological tests were performed as a germination or viability test to establish the invasive capacity of B. bassiana, and purity and spore concentration tests to determine the microbiological quality of the two formulations.

e. Germination or viability test

The viability of the fungus was established in combination with an estimate of the number of spores per gram of rice by calculating the number of viable spores per unit of weight or volume. This test was performed from dilution 10-3 in 3% agar-water inoculating 5µl, at 5 points in the medium. The inoculated petri dishes were incubated at 27°C for 7 days. After this time, the agar corresponding to each one of the aliquots was extracted and placed in slides with blue lactophenol. The observation was made under the microscope with the objective of 40x counting a minimum of 100 spores between germinated and not germinated, for each aliquot and the percentage of germination was calculated.

f. Spore concentration test

The number of infective units per weight unit in each formulation was determined. The mother suspension was prepared in a test tube by adding 1 gram of rice with the entomopathogen B. bassiana and sterile distilled water (ADE) with tween 80 to 0.1%, until completing 10 ml. Four subsamples of 1 ml were taken and deposited in tubes with 9 ml of sterile distilled water (SDA), being prepared dilution 10-1 from which other dilutions up to 10-5 were prepared. The spore count was carried out in the neubauer chamber or hematocytometer [8]. The C spore concentration was calculated by multiplying the average number of spores per quadrant obtained (N) by the inverse of the dilution used and the chamber factor. C= N x inverse of the dilution used x Factor of the chamber (5x104). This concentration was expressed in spores/g (e/g). This value corresponds to the estimated number of spores per gram of rice or spores per milliliter.

$$C = \sum(A + B + C + D + E) * 50000 * FD = \frac{\#sporas}{gram\ of\ rice} \quad (1)$$

g. Purity test

It was carried out to establish the percentage of the biological agent in the formulation taken and to identify the contaminants, helping to improve the production process of the entomopathogen. The Potato Dextrose Agar (PDA) plus 1 µl of ampicillin was used. After agitation of the dilution tubes in the vortex, 50 µl were taken to inoculate in each petri dish, dispersing with a hockey handle, the boxes were incubated at 27° C. The number of Colony Forming Units (CFU) of each organism was counted daily for 5 days. At the end of the readings, the number of B. bassiana CFU and the number of other microorganisms (fungi, bacteria and yeasts) were recorded. For the calculation of purity (P) the following formula was used:

$$\% P = \frac{CFU\ of\ the\ desired\ fungus\ x\ 100}{total\ CFU} \quad (2)$$

h. Pathogenicity test: Bioessays

The larvae were selected considering the macroscopic characteristics of the O. cassina larval instar III and transferred to the laboratory in glass jars with a capacity of 3L at room temperature. For the development of the bioassay, 10 larvae were arranged per duplicate treatment for a total of 60 larvae. Each treatment was fed fresh oil palm foliage with an inter-day frequency [1].

To establish the infective capacity of B. bassiana, the formulations were applied to each of the treatments (control group (without application), solid formulation, liquid formulation) at a concentration of 1010 spores/ml by spraying, following the indications of Cenicafé [9]. Live and dead larvae were then monitored. The readings ceased when the mortality of the larvae treated with B. bassiana occurred. The dead larvae were extracted from the vials and placed in a wet chamber in a MICOSEL medium, where they were kept for 8 to 12 days, the chambers were checked every two days to observe the time in which the sporulation occurred, after macroscopically observing the growth of B. bassiana, microscopic observation with lactophenol blue was performed.

III. RESULTS, ANALYSIS AND INTERPRETATION

The results obtained in the quality tests for spore concentration, spore germination and the viability and microbiological purity of the evaluated formulations used for the pathogenicity test on Opsiphanes cassina are presented in Table 1.

a. Invasive capacity in vitro of B. bassiana using viability tests of the fungus

According to Shah [10] entomopathogenic fungi as biological control agents are seen as promising microbial agents for the control of insect pests and are becoming an interesting alternative to chemical control, such is the case of B. bassiana which presented 90% germination or viability in the solid formulation, while in the liquid formulation obtained 97.7%. These results were above those reported by Posada et al., [11] who refer to germination percentages of 86.1%, which indicates that the percentage of germination in both the solid and liquid formulation obtained in this research is adequate, since germination depends on the conidia absorbing moisture from the medium to maintain the viability of the fungus. Góngora et al., [9] who affirm that the most important quality tests are viability and virulence, since if a spore formulation has high viability and virulence, it is more likely to act well in the field to control the insect. Therefore, it was demonstrated that formulations based on B. bassiana can cause infection and mortality to the larval stage of O. cassina evaluated in this paper.

Table 1. Quality testing of evaluated formulations

Formulation	Spores concentration spores/g	Spores germination %	Purity %	Pathogenicity %	Observations
Liquid	$8.6x10^{10}$	97,7	100%	100%	Mortality 6 days after application of the product
Solid	$4.9x10^{10}$	90	99,5%	100%	Mortality 8 days after application of the product

Source: Prepared by the authors.

b. Microbiological quality of evaluated formulations

Gómez and Mendoza [7], state that the purpose of the purity test is to establish the proportion of the biological agent and identify contaminants, helping to improve the entomopathogen production process. At the national level, resolution 000698 of 2011 [12], an entomopathogenic product or bio input for agricultural use must have a purity of ≥95% and must not contain contaminating microorganisms or pathogens to humans, plants and animals. Therefore, the results of this research allowed corroborating the purity of the fungus corresponding to 100% in the liquid product and 99.5% in the solid; indicating that either the two of the evaluated formulations is in adequate conditions for application at both laboratory and field level.

Another test to determine the quality of the entomopathogenic product is the spore concentration. Studies carried out by Gómez and Mendoza [7], show that the adequate spore concentration is 8 x 108conidias/g rice. According to this, it is considered that the concentration of spores of the fungus B. bassiana in the formulation both solid and liquid, corresponding to 4.9x1010 conidia/g rice and 8.6x1010 conidia/ml respectively, comply with this criterion, which could guarantee the death of the insect in a few days as referred to by Hajjar et al. [13], who with concentrations lower than 1x108 spores/ml, recorded the first death after 4 days of direct application.

It should be noted that under adequate conditions of temperature and humidity adequate concentrations of B. bassiana can be obtained to be used as biological control. According to Cenicafé, when B. bassiana is cultivated. B.bassiana Bb9205 in rice, under controlled conditions in the laboratory, concentrations of up to 1x109 spores/g of rice can be obtained [9].

a. Infective capacity of Beauveria bassiana through pathogenicity bioassays

It was demonstrated that the conidia of B. bassiana act mainly by contact, when the fungus is able to penetrate the insect and invade it, causing it to die from mycosis. According to Téllez [6], death can occur within 3 to 5 days. Given the above, in this investigation it was possible to confirm a greater mortality 6 days after the application of the liquid product corresponding to 100%, regarding the solid product that registered a mortality of 100% 8 days after its application, this guarantees that the entomopathogenic fungus is the cause of the mortality of the insect pest, given that the pathogenicity tests of commercial products must cause mortalities higher than 80% [9].

Table 2. Pathogenicity Bioassays

	Solid formulation				Liquid formulation				Control		
Day	**LL**	**%**	**DL**	Day	**LL**	**%**	**DL**	Day	**LL**	**%**	**DL**
1	10	100	0	1	10	100	0	1	10	100	0
2	10	100	0	2	10	100	0	2	10	100	0
3	7	70	3	3	7	70	3	3	7	70	3
4	4	40	3	4	4	40	3	4	4	40	3
5	2	20	2	5	2	20	2	5	2	20	2
6	2	20	0	6	2	20	0	6	2	20	0
8	2	20	0	8	2	20	0	8	2	20	0
9	0	0	2	9	0	0	2	9	0	0	2

Source: Prepared by the authors.
LL: Live larvae DL: Dead larvae

According to Zhang, L. et al., [14], the pathogenicity of B. bassiana Bb1801 in the larvae of the red turpentine beetle, Dendroctonus valens, killed 100% of the treated larvae in approximately 4.6 days at a concentration of 1x107 spores/ml. In the present study it was observed the death of the larvae of the III instar of O. cassina from 48 hours, the duration is due to the relation that exists between the time and the contact that occurs between the larvae and their infection directly or indirectly by B. bassiana. Studies performed by Hajjar, M. et al., [13], showed the first death caused by the studied concentrations at 1x108, 1x107 and 1x106 spores/ml of B. bassiana at 4, 6 and 9 days after treatment, respectively. Given the above, there is a similarity between the mortality times of O. cassina larvae and other studies in which B. bassiana is used as biological control of different pests. It should be noted that an adequate temperature and humidity are important to maintain the viability of the fungus. The previous evidences are related with the results obtained in this study in humid chamber observing growth on the larvae from 5 to 12 days, and it was confirmed with the presence of its microscopic characteristics.

IV. CONCLUSIONS

Taking into account the results of this investigation, it can be concluded that the liquid presentation of the product based on Beauveria bassiana showed a higher efficiency given mainly by the higher concentration of spores of the fungus, infective capacity, pathogenicity in a shorter time and reduction in the time of obtaining the product showing its feasibility for a massive production and possible commercialization of it.

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