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Evaluation of *Beauveria* sp strains, conidial concentration and immersion times on mortality rate of bovine tick (*Boophilussp*).

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ABSTRACT

Ticks (*Boophilus* sp) are one of the main problems that affect **the**performance of bovine production in tropic and sub-tropical. **Organophosphate acaricides are the usual tick control method**,however the ecological damage and cross-resistance**to those acaricides is**leading **to the** search **for** alternatives. *Beauveria bassiana* can be used **as a** biological control, however, **laboratory bioassays with ticks showed mortality of 20 to 50% only**. The aim of this investigation was to study the effect of five strains of *Beauveria sp*, two conidial concentrations and three immersion times on mortality rate of ticks. Five strains of *Beauveria sp* isolated in México were used and cultivated in liquid medium until 10^7 and 10^8 conidia/mL were obtained. For *in vitro* bioassays, ticks were collected from bovine, immersed in conidial suspensions for 20, 30 and 40 seconds. Mortality rate was recorded daily up to 10 days.Strain type was the main factor and 23-D *Beauveria*strain **hadthe highest** value (100%) of mortality rate after ten days of incubation with ticks. The **best** immersion time was 20 s and the **best**conidial concentration was 10^8 conidial/ml. In conclusion, native *Beauveria sp* strains could be an alternative viable for ticks control in bovine, however field test and Beauveria identification are necessary.

1. INTRODUCTION

Internal and external parasites of cattle remain one of the main causes of economic losses in Latin America and other livestock regions of the tropics and subtropics of the world [1]. Over the past four decades the development of miticides, insecticides and anthelmintics highly effective withbroad spectrum and residual power, has allowed the farmer to control these plagues. However, instead of solving the problem, they settled insecticides to kill both natural predators and pests [2]. The death of natural predators caused other insect plagues that used to be under control, increasing their populations significantly. Many species of insects considered plagues have developed resistance to one or more insecticides [3].The presence of ticks is one of the significant problems that affect performance in livestock production units [4]. However, the levels of resistance to two organophosphate acaricides, coumaphos and diazinon, in several Mexican strains of *Boophilus microplus* (Canestrini) were evaluated and data revealed a significant cross-resistance pattern between those two acaricides [5]. Therefore the search for alternative control with an emphasis on natural controls and lesser ecological damage has been stimulated. An alternative is the use of entomopathogenic fungi (EF) such as Metarhizium, Beauveria, Paecilomyces, Verticillium, Fusarium and Rhizopus [6]. In particular, *B. bassiana* has been proved to be an efficient and environmentally friendly biocontrol agent against a variety of plagues [7, 8, 9]. Unlike other microorganisms, *B. bassiana* does not need to be ingested to exert their pathogenicity, and that penetrate through cuticle due to its ability to excrete enzymes (proteases, kinases, lipases, lipoxygenases) [10]. It has been reported that *B. bassiana*can be used for biological control of the cattle ticks, however, laboratory bioassays showed a mortality of only 20 to 50% of the ticks seven days after inoculation with 10^7 *Beauveria* conidia/mL. Mortality

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values differed for each Beauveria strain tested [11]. Therefore; the aim of this investigation was to study the effect of five different strains of *Beauveria sp*, two conidial concentrations, three immersion times and ten incubation days on mortality rate of ticks (*Boophilus microplus*).

2. MATERIALS AND METHODS

Five strains of *Beauveria* sp were used. Bb-19 strain was donated from the Mycological collection of El Colegio de la Frontera Sur (ECOSUR), Chiapas, Mexico, Tapachula unit; while the 23D, 12.11, 12.15 and 12.05 strains were donated from the Mycological collection of the National Research Institute of Forestry, Agriculture and Livestock (INIFAP-General Teran), Nuevo Leon, Mexico.

Inoculation was made from a Petri dish containing pure strains of *Beauveria* sp. with 10 days of active growth in Agar potato medium. The preparation of liquid medium was done using 250 mL Erlenmeyer flasks with 50 mL of liquid medium containing minerals ($NaNO_3$, K_2HPO_4, $MgSO_4$, KCL, and $FeSO_4$), vegetable juice (celery, watercress, beets, spinach, tomatoes, lettuce, parsley and carrot), chitin 1% and Tween 80, the flasks were autoclaved (121°C, 15 lb/inch^2 for 15 min). Inoculation with B. sp was performed in laminar flow chamber previously disinfected with 70% alcohol. Flasks were kept under constant stirring at 180 rpm for 3 days at room temperature, where a concentration of 10^7 and 10^8 conidia / mL were obtained. The counting of conidial was done according to Cañedo[12]. Briefly, a sample of liquid medium was taken with a pipette; a small drop was placed on each of the deposits of the Neubauer chamber. The concentration of conidial was calculated as follows:

$$NC = \left(\frac{SC}{5}\right) * 25 * 50{,}000$$

Where:

NC = number of conidial per milliliter of suspension.

SC = Sum of conidia contained in the five pictures from the Neubauer chamber.

2.1 Collection of ticks in animals.

Ticks were collected directly from bovine animals, making a previous interview of the production unit in order to obtain information regarding the background to the use of external antiparasitic. Subsequently we carried out the collection of ticks with a simple random method. We randomly sampled 30 animals and we collected 30 adult ticks for identification post. The selected animals were handled individually, and subsequently underwent a rigorous visual physical examination and palpation of the skin, to detect ticks at any stage. The examination was performed systematically in pre anatomical regions: Pavilion ears, head, neck, back, earlier train, underarms, chest, belly, udder, scrotum, flanks, groin, hindquarter, perineum and tail. Ticks were deposited in test tubes with cotton plugs. Ticks were transported to laboratory of Parasitology in the Faculty of Veterinary Medicine at the Autonomous University of Chiapas (FMVZ -UNACH). Adult ticks were placed in glass vials with cotton plugs to allow breathing, incubated at 27° ± 2° C and with 80-85% relative humidity (RH) to stimulate oviposition. Eggs were placed in glass vials covered with filter paper under the same conditions of temperature and relative humidity. Larvae 14 days old were used in bioassays for the evaluation of pathogenicity of *Beauveria* sp. For the pathogenicity evaluation of the isolated strains of the fungus *B. sp* on populations of ticks, a bioassay was installed under controlled laboratory conditions (temperature and relative humidity) using a factorial design 5 x 3 x 2 completely randomized with 30 treatments and four replications for each one. To calculate the mortality rate, 20 larvae of ticks were immersed in *Beauveria.* sp suspensions with 10^7 and 10^8 conidia/ml for 20, 30 and 40 seconds and then placed in a paper towel to absorb excess of liquid medium. The control group was treated with sterile water and 1% Tween 80 in place of the suspension of conidia.

2.2 Statistical analysis

The effect of treatment was determined by analysis of variance with a significance level of 5% and the mean test was conducted by Media Significant Difference test. The Statgraphic Plus (Statgraphics Centurion XV, 2007) was used for the regression analysis of the experimental data.

3. RESULTS AND DISCUSSIONS

In Table 1 are presented the different treatments according to the experimental design factorial complete 5 x 3 x 2, results of mortality percentage of ticks isolated from bovine exposed to different *Beauveria* sp strains, immersion times and conidial concentrations. Mortality percentage of *Boophilus* sp varied from 60 to 100 after ten days of incubation time (Table 1).

ANOVA for main effects and interactions of strain, conidial concentration and immersion time *Bauvaeria* sp on mortality percentage of ticks are presented in Table 2. Straintype of *Beauveria* sp and interactions had a significant effect on mortality rate of tick. In relation with the strain type, the 23-D strain had the highest value (100%), whereas the lowest value (82%) of mortality rate of tick (*Boophilus* sp) was obtained with 1211 strain after ten days of incubation (Table 3). The effects of *Bauvaeria* sp strain 23-D on mortality percentage of tick (*Boophilus* sp) started after two day of incubation and gradually increased until it reaches 100% of mortality (Figure 1).

Strain type of *Beauveria* sp and interaction between strain type, immersion time and conidial concentration had a significant effect on mortality rate of tick. 23-D strain had the highest value (100%), whereas the lowest value (82%) of mortality rate of tick (*Boophilus sp*) was obtained with 1211 strain. These results could be explained for differences among enzyme activities in *Beauveria* strain type and it is necessary the characterization of certain enzymes such as phosphoenolpyruvate carboxykinase, isocitratelyase and pyruvate carboxylasein each strain. These enzymes have been recognized as vital components of several biochemical reactions in other fungi and bacteria [3].

Table 1: Experimental design factorial complete 5 x 3 x 2 and results of mortality percentage of ticks isolated from bovine exposed to different *Beauveria* sp strains, immersion times and conidial concentrations. Evaluation was done after ten days of incubation days and results are media of five repetitions.

Treatment	Strain	Immersion time	Conidial concentration	Mortality
		—s—	—Conidial/ml—	—%—
1	19	20	7	80
2	19	20	8	90
3	19	30	7	80
4	19	30	8	90
5	19	40	7	90
6	19	40	8	90
7	1205	20	7	90
8	1205	20	8	90
9	1205	30	7	95
10	1205	30	8	95
11	1205	40	7	95
12	1205	40	8	90
13	1211	20	7	90
14	1211	20	8	80
15	1211	30	7	80
16	1211	30	8	80
17	1211	40	7	100
18	1211	40	8	60
19	1215	20	7	90
20	1215	20	8	100
21	1215	30	7	90
22	1215	30	8	100
23	1215	40	7	90
24	1215	40	8	100
25	23D	20	7	100
26	23D	20	8	100
27	23D	30	7	100
28	23D	30	8	100
29	23D	40	7	100
30	23D	40	8	100
control				0

Table 2: ANOVA for main effects and interactions of strain, conidial concentration and immersion time *Bauvaeriasp* on mortality percentage of tick (*Boophilus sp*). Data used were obtained after ten days of incubation.

Source	Square sum	Df	Mean square	F- reason	P- value
Main effect					
A:strain	6151.67	4	1537.92	128.89	0.0000
B:Inmmersion time	16.0	2	8.0	0.67	0.5133
C:Conidial concentration	0	1	0	0.00	1.0000
Interactions					
AB	337.333	8	42.1667	3.53	0.0010
AC	3201.67	4	800.417	67.08	0.0000
BC	916.0	2	458.0	38.38	0.0000
Residual	1527.33	128	11.9323		
TOTAL (corrected)	12150.0	149			

All-F reasons are based on the mean square of residual error

The effects of *Bauvaeria sp* strain 23-D in mortality percentage of tick (*Boophilus sp*) started after two day of incubation and gradually increased until it reaches 100% of mortality. This kinetic pattern could be explained because *Beauveria bassiana* infects insects by direct cuticle penetration rather than by ingestion or through a wound like viruses or bacteria [13]. The infection process consists **in** three stages: 1) attach to the cuticle, 2) penetrate the cuticle, and 3) proliferate in the haemocoel and kill the host. The whole process is rather complex and multiple host factors and fungal toxins could be involved in the process [14]. Although insect resistance to *B. bassiana* has not yet been reported, some disadvantages impede the wide use of fungal biological agents. For example, they are not as fast acting as chemical insecticides and their efficacy varies with field conditions. Successful application of *B. bassiana* needs favorable environmental conditions such as high humidity and medium temperature. Recently Xiao [10] sequenced the genome of *B. bassiana* and a phylogenomic analysis confirmed that ascomycete entomopathogenicity is polyphyletic, but also revealed convergent evolution to insect pathogenicity. *B. bassiana*is having more bacterial-like toxins (suggesting an unsuspected potential for oral toxicity) and effector-type proteins. A high throughput RNA-seq transcriptomic analysis revealed that *B. bassiana* could sense and adapt to different environmental niches by activating well-defined gene sets [10].

In our study, the better strain was the 23-D, donated for Mycological collection of the National Research Institute of Forestry, Agriculture and Livestock (INIFAP) Mexico. It will be necessary to characterize this strain both genetically and metabolically.

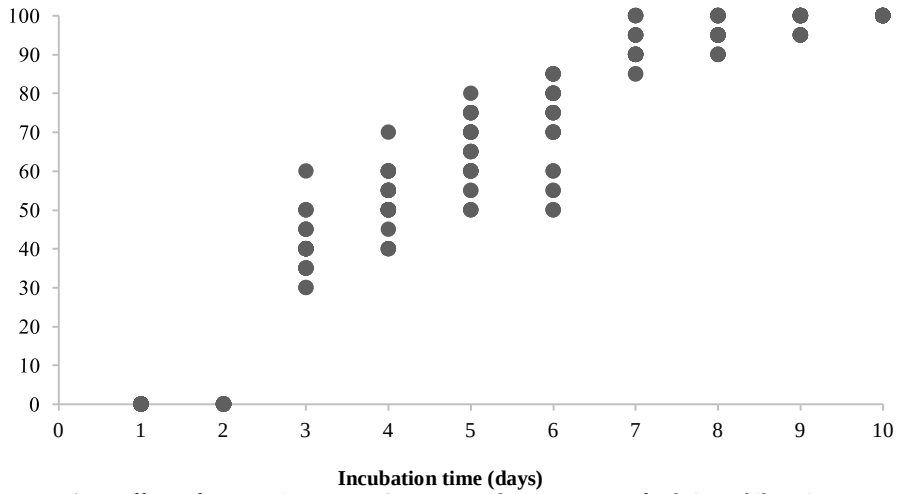

Fig 1: Effects of *Bauvaeriasp* strain 23-D on mortality percentage of tick (*Boophilus* sp).

Table 3: Evaluation of strain, conidial concentration and immersion time of *Bauvaeriasp* on mortality percentage of tick (*Boophilus* sp).

			Standard	**Homogeneous**	***Lower***	***Upper***
Factors	*observations*	*Media*	*Error*	group	*limit*	*Limit*
strain						
1205	30	91.8	0.63	c	90.58	93.08
1211	30	81.7	0.63	e	80.41	82.91
1215	30	95.0	0.63	b	93.75	96.24
19	30	86.5	0.63	d	85.25	87.74
23D	30	100.0	0.63	a	98.75	101.28
LSD (0.05)		1.76				
immersion time						
20	50	91.0	0.49	a	90.03	91.96
30	50	90.6	0.49	a	89.63	91.56
40	50	91.4	0.49	a	90.43	92.36
LSD (0.05)		1.37				
conidial concentration						
1 x 10^7	75	91.0	0.39	a	90.2108	91.7892
1 x 10^8	75	91.0	0.39	a	90.2108	91.7892
LSD (0.05)		1.12				

[a]Values are media calculated with all incubated days and two conidial concentrations. [b]Values are media calculated with all strains and all incubation days.
[c]Values are media calculated with all strain and two conidial concentrations.

4. CONCLUSION

Beauveria sp can be used for controlling ticks in bovine, however the effects depend heavily on the strain used rather than the conditions of application of the fungus. Additional field tests and *Beauveria* sp identification are necessary to obtain data that can support the commercial application of this method of biological control of ticks

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