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*Full Length Research Paper*

# Study of *Beauveria bassiana* growth, blastospore yield, desiccation-tolerance, viability and toxic activity using different liquid media

**Mabelle Julie Chong-Rodríguez[1], María Guadalupe Maldonado-Blanco[1]*, Jesús Jaime Hernández-Escareño[2], Luis Jesús Galán-Wong[1] and Carlos Francisco Sandoval-Coronado[1]**

[1]Instituto de Biotecnología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León. Av. Pedro de Alba y Manuel L. Barragán s/n Ciudad Universitaria, San Nicolás de los Garza, Nuevo León, C. P. 66450, A. P. 414 and 2790. México.
[2]Departamento de Microbiología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Nuevo León. Av. Pedro de Alba y Manuel L. Barragán s/n Ciudad Universitaria, San Nicolás de los Garza, Nuevo León, C. P. 66450, A. P. 414 and 2790. México.

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***Beauveria bassiana*** **was grown on three liquid media containing casaminoacids, corn steep liquor or peptone. After incubation, the blastospore counts reached 6.38 × $10^9$ blastospores/ml, in the medium containing sucrose and corn steep liquor, which was significantly higher than the obtained with media containing casaminoacids or peptone. The medium containing corn steep liquor produced predominately submerged conidia, meanwhile the other media produced blastospores. The blastospores produced in the medium containing casaminoacids presented faster germination rates, than the blastospores produced in media containing corn steep liquor or peptone, although, after air-drying, were observed significant reductions on the viability of blastospores produced in the media composed by casaminoacids or peptone, but the spores produced in the medium with corn steep liquor were not affected. For storage of blastospores at 4 and 26°C after some months, the blastospores produced in the medium with casaminoacids showed the highest viability at 26°C, whereas at 4°C the counts of viable blastospores produced in medium containing corn steep liquor were significantly higher than the counts of blastospores produced in the other media with casaminoacids or peptone. The blastospores maintained for six months at 4°C showed high mortality against third-instar *Plutella xylostella* larvae.**

**Key words:** *Beauveria bassiana,* liquid media, fermentation, short times of propagation, biological control, entomopathogen fungus.

## INTRODUCTION

*Beauveria bassiana* is an imperfect entomopathogenic fungus that attacks a wide range of agricultural pests (Feng et al., 1994) and also grows on soil as saprophyte (Bidochka et al., 1998). Liquid-culture production of *B. bassiana* blastospores is a suitable biotechnological process from the point of view of efficiency and profitability, when compared with the production of spores on solid-culture surfaces or using two-phase fermentation. However, some times is necessary to use these two-phase fermentations in order to obtain cells with active synchronic growth to inoculate media for studies of optimization.

The mass propagation of fungi for use as biopesticide is a goal for investigators, because include advantages as mainly on the processes of scaling which are relatively easy, as well as the control of parameters such as temperature, aeration and pH. In addition, production costs can be lower.

*Corresponding author. E-mail: mgpemald@hotmail.com, mgmb_uanl@yahoo.com.mx. Tel: +528183294000 ext. 6464. Fax: +528183294000 ext. 6415.

However, some of the propagules obtained in liquid culture are very unstable under environmental conditions. The fungus loses viability at low humidity and UV light exposure (Luz and Fargues, 1999). Then, the capacity to infect the host is reduced. This is the reason why some studies are focus on nutritional aspects of fungus production, in order to develop endogenous reserves that allow the fungus to resist adverse conditions (Lane et al., 1991).

Some studies made with *B. bassiana* reveal that, the carbon sources used for production are closely related with the spore production (Thomas, 1987) and also with the spore-type produced (Hegedus et al., 1990), whereas Jackson et al. (1997) demonstrated that, the adequate sources of carbon and nitrogen in the culture media, would produce tolerant-desiccation blastospores of *Isaria fumosorosea* after air-dried conditions; in a similar way, Sandoval-Coronado et al. (2001) found that, different supports used for formulation, such as talc, lime, gypsum or clay maintained the viability of *I. fumosorosea* propagules to levels around 50 to 70% for cultures obtained in liquid media after different storage times.

In this study, we evaluated the effect of different carbon and nitrogen sources on the growth, blastospore yield, desiccation-tolerance of blastospores, viability and toxic activity of *B. bassiana* grown in submerged culture.

## MATERIALS AND METHODS

### Strain

The GHA *B. bassiana* strain, was obtained from USDA ARS (Peoria, Illinois) then, was activated as single-spore isolates on potato dextrose agar (PDA), incubated for two weeks at 26 ± 1°C after the conservation for long time was made placing 1 $cm^2$ little-squares into 10% (v/v) glycerol vials at -80°C. All isolates were routinely cultured at same temperature (26 ± 1°C).

### Inoculum and propagation

Conidial suspensions were prepared by flooding 2-weeks-old PDA Petri dish cultures of the strain using 0.1 % v/v Tween 80 sterile solution. The *B. bassiana* cultures provided a final concentration of $1 \times 10^7$ spores /ml which was used as inoculum (10 ml) of each 90 ml culture media contained in a 250-ml Erlenmeyer baffled flask. The flasks were incubated at 26 ± 1°C temperature and 300 rpm agitation in a rotary shaker for 3 days (pre-culture). After, for culture studies the inoculum was adjusted at $1 \times 10^8$ blastospores/ml using 250 ml baffled flasks, which were incubated as mentioned earlier for three days.

### Media composition

Three different liquid media were used which contained different sources of carbon and nitrogen. The medium M1, reported by Jackson et al. (1997) contained per litre: $KH_2PO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $CaCl_2 \cdot 2H_2O$, 0.4 g ; $FeSO_4 \cdot 7H_2O$, 50 mg; $CoCl_2 \cdot 6H_2O$, 37 mg; $MnSO_4 \cdot H_2O$, 16 mg; $ZnSO_4 \cdot 7H_2O$, 14 mg; thiamin, riboflavin, pantothenate, niacin, pyridoxamine, and thioctic acid, 500 µg each; biotin, folic acid and vitamin $B_{12}$, 50 µg each; glucose 80 g and vitamin-free casaminoácidos 15 g (pre-culture) and 25 (culture). The composition of T2 and T3 media per litre was as following: $KNO_3$, 10 g; $KH_2PO_4$, 5 g; $MgSO_4$, 2 g; $CaCl_2 \cdot 2H_2O$, 0.8 g; $FeSO_4 \cdot 7H_2O$, 50 mg; $CoCl_2 \cdot 6H_2O$, 37 mg; $MnSO_4 \cdot 2H_2O$, 16 mg; $ZnSO_4 \cdot 7H_2O$, 14 mg. The medium T2 contained additional 50 g glucose (preculture), 50 g sucrose (culture) and 20 g corn steep liquor, whereas the medium T3 contained 50 g glucose and 50 g peptone. Glucose and sucrose were autoclaved separately. Four replicate flasks were used for each treatment and all experiments were repeated at least thrice. All components of the media were obtained from Difco Laboratories, Detroit, MI, USA.

### Drying and viability

The blastospores obtained in liquid culture were dried at 26 ± 1° C, using an air-drying method which consisted of mixing the cultures with 5% (w/v) diatomaceous earth (HYFLO®, celite corp., Lompoc, CA, USA) then, the suspension was vacuum-filtering using a Whatman # 1 filter paper to remove the excess liquid. After that, the viability test was assessed by germination of blastopores in Sabouraud broth (Difco). For this test, 100 mg of the powder containing the blastospores was placed into 250 ml-flasks containing 50 ml of the broth and then, was incubated under agitation at 300 rpm and 26 ± 1°C. Samples were taken before and after drying at 2, 4, 6, 8, 10, 12 and 14 h in order to determine the desiccant-tolerance percentage. The germination of blastospores was assessed by counting a group of 100 blastospores per aliquot, observed through light microscope and the viable blastospores were recognized by the presence of germ tube of half-blastospore size.

### Storage studies

For this test, the air-dried blastospores in diatomaceous earth were kept in sealed plastic bags at temperatures of 4 or 26 ± 1°C for six months. For long term storage studies, the spore survival was measured by viable plate counts on potato dextrose agar (PDA) Petri dishes (triplicates) incubated at 25°C until the colonies became visible at 3 to 4 days of incubation. The counts were registered on the appropriate dilution (30 to 300 colonies) and the averages were obtained.

### Bioassay

The biological activity of air-dried blastospores on diatomaceous earth was determined through bioassays against *Plutella xylostella* three-instar larvae. The preparations maintained during six months at 4°C, were mixed in water containing 0.1% (v/v) Tween 80. These concentrations of spores applied were measured and adjusted to $1 \times 10^8$ spores/ml using the haemotocytometer and the viable spores were determined by viable plate counts in PDA. Ten larvae were submerged for 30 s in the suspensions and placed after into wet filter paper over 1.5% agar-water Petri dishes (triplicates). In the experiment, was included a positive control that consisted of a suspension of *B. bassiana* aerial conidia obtained in potato dextrose agar Petri dish, adjusted to $1 \times 10^8$ conidia/ml and an untreated control that was sterile distilled water containing only 0.1% Tween 80 and a second untreated control containing water and diatomaceous earth (triplicates). The treated larvae and controls were kept for eight days at 25°C, 70% relative humidity and 14:10 h photoperiod.

### Statistical analysis

Data analysis was performed using one-way ANOVA, for each

**Table 1.** Production of GHA *B. bassiana* blastospores in three different culture media with variable sources of carbon and nitrogen fewer than two growth stages.

| Medium | Growth stages ( spores/ml ± S.E[a]) | |
|---|---|---|
| | Pre-culture | Culture |
| M1 (Casaminoacids) | $7.2 \times 10^8 \pm 2.1 \times 10^8$ | $1.76 \times 10^9 \pm 1.12 \times 10^9$ |
| T2 (Sucrose and corn steep liquor) | $1.7 \times 10^9 \pm 4.2 \times 10^8$ | $*6.38 \times 10^9 \pm 3.63 \times 10^9$ |
| T3 (Glucose and peptone) | $1.8 \times 10^9 \pm 8.6 \times 10^8$ | $1.34 \times 10^9 \pm 0.611 \times 10^9$ |

[a]Standard error of mean; * indicate significant difference at p≤ o.05

determination, including growth, yield blastospores, storage and biological activity and the means were compared on each case using the least significance difference (LSD) method at 0.05 level, whereas a t-test (Student) was used to compare the germination rate before and after of air-drying blastospores for the desiccation tolerance.

## RESULTS AND DISCUSSION

### Yield

Different types of spores and different levels of production were obtained with the three liquid media. Evaluation of spore morphology indicated that, medium containing corn steep liquor (T2) produced predominately submerged conidia, while media supplemented with casaminoacids (M1) or peptone (T3), produced blastospores after 6 days of fermentation. The results obtained from the growth of *B. bassiana* into the three different culture media tested are shown in the following Table 1, whereas it was found that the best production of spores was obtained in the medium T2, which contained glucose in the preculture and sucrose and corn steep liquor in the culture medium and was obtained an average of $6.38 \times 10^9$ blastopores/ml, which was significantly higher than the obtained with the other two media, M1 and T3 ($F= 9.8351$ df = 2, 6, $P \leq 0.05$).

These results of production of spores were higher than the obtained by other investigators, in studies of liquid fermentation of entomopathogen fungi like the reported by Thomas et al. (1987) of $5 \times 10^8$ spores/ml for *B. bassiana*, as well as Jackson et al. (1997), who found $6.8 \times 10^8$ spores/ml for cultures of *I. fumosorosea* and from Sandoval-Coronado et al. (2001), who reported $5 \times 10^8$ spores/ml, for this same strain. This result obtained in the medium T2 suggests that, the corn steep liquor and sucrose could be good nitrogen and carbon sources for high spore production of GHA *B. bassiana*. For the formulation of the medium T2, we intentionally used corn steep liquor and sucrose because these ingredients were reported to be a good source for blastospore production (Bláchere et al., 1973; Samsináková, 1966), but we obtained submerged conidia in our fermentation, this result is in accordance with Yin et al. (1988) who reported that, the use of corn steep liquor increases the production of submerged conidia.

The spore production is closely related with the oxygen rate and carbon sources (Feng et al., 1994) and also the nitrogen sources are very important because this is one of the main components of microorganisms (Zabriskie et al., 1999). Various studies have demonstrated the influence of nitrogen sources like: peptone, yeast extract (Hegedus et al., 1990); corn steep liquor (Samsináková, 1966); $(NH_4)_2 SO_4$ (Lane et al., 1991) and casaminoacids (Jackson et al., 1997) in the production of blastospores.
The amount of blastospores of GHA *B. bassiana* obtained by us in the medium M1 ($1.76 \times 10^9$) was very similar to those reported by Jackson et al. (1997) for blastospores production of *I. fumosorosea*, in this same medium, in addition, we found blastospore production in the medium containing peptone (T3); these results were a confirmation to the studies made by Bidochka et al. (1987), where they used peptone and yeast extract as nitrogen sources and obtained good blastospore production.

### Desiccation tolerance and viability of *B. bassiana* spores

The germination rates of blastospores produced were measured before and after air-drying from 0 until 14 h, to determine differences between the three media tested here. The blastospores produced in the medium M1 presented faster germination rates, than the blastospores produced in media T2 and T3, although, after air-drying the desiccation tolerance was different for each culture.

The blastospores produced different germination percentages at different times. The higher germination percentage was observed at 10 h (75.8), for the blastospores produced in M1. The blastospores produced in T2 presented 73.7% germination and finally, the blastospores produced in T3 showed 60% (Figure 1). After air-drying, the germination rate decreased to 35.2, 64.5 and 21.6% for the blastospores grown in M1, T2 and T3, respectively (Figure 2).

These values were analyzed through a t-test (Student) for determine differences, before and after air-drying, were found significant reductions in the germination percentage for the blastospores produced in the medium M1 ($t$ calculated = 10.53, df = 8, $t_{0.05} = 2.30$, $t_{0.01} = 3.35$) and T3 ($t$ calculated = 5.57, df =8), but the blastospores

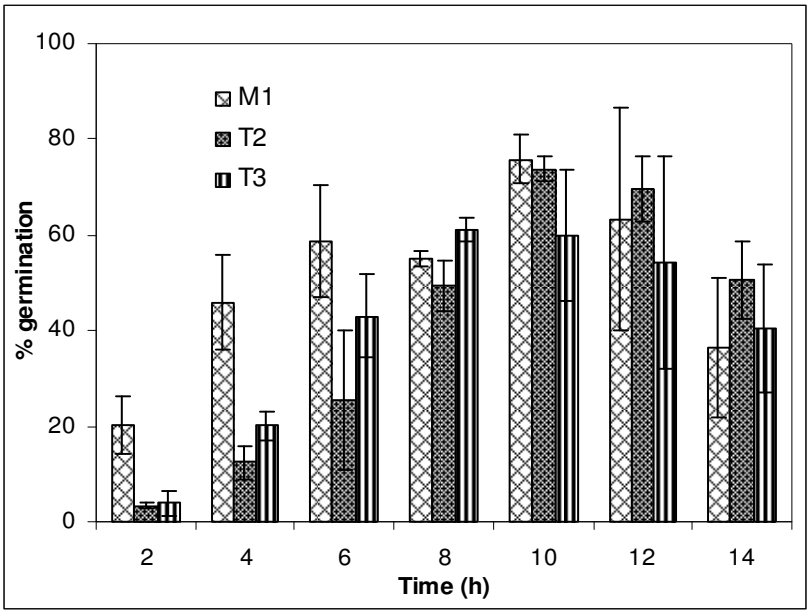


**Figure 1.** Spore germination rate of GHA *B. bassiana* blastospores before air-drying at 26 ± 1°C, grown in the media M1 (casaminoacids), T2 (corn steep liquor) and T3 (peptone).

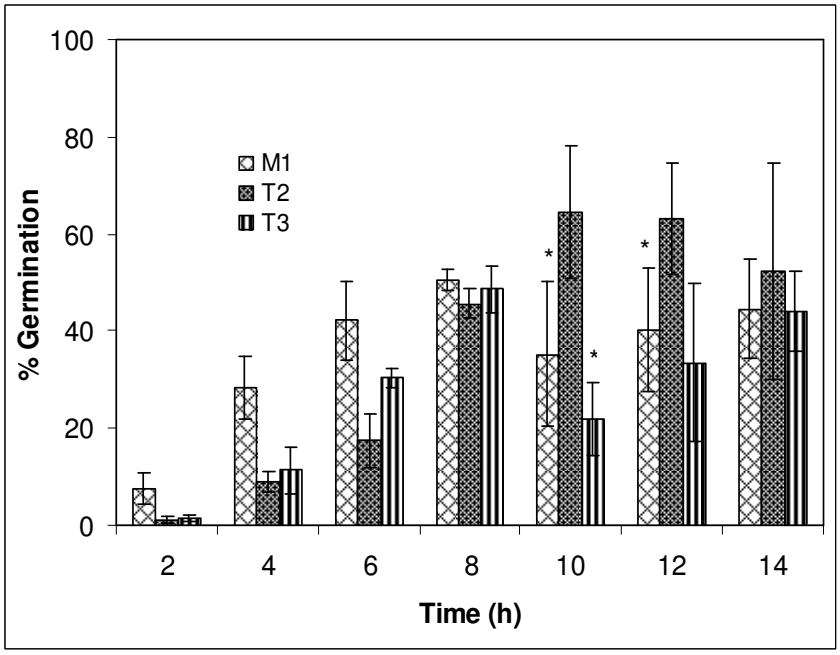


**Figure 2.** Spore germination rate of GHA *B. bassiana* blastospores after air-drying at 26 ± 1°C, grown in the media M1, T2 and T3. Asterisks indicate significant difference between number of blastospores before and after air-drying and analyzed through a t-test (Student) at 0.05 level.

produced in the medium T2 not were affected (t calculated = 1.98, df =8). At similar way, the average germination results for 12 h after air-drying were compared and significant reductions were found for the blastospores produced in the medium M1 only (t calculated = 6.33 df = 8), whereas for 14 h after air-

**Table 2.** Viable counts of GHA *B. bassiana* blastospores produced in media M1, T2 and T3 after air-drying and formulated with diatomaceous earth during storage by six months at 4 and 26°C.

| Medium | 4°C | | | | | |
|---|---|---|---|---|---|---|
| | 1 month | 2 months | 3 months | 4 months | 5 months | 6 months |
| M1 | 51 ± 15 | 66.8 ± 33.1 | 44.6 ± 36.1 | *50.5 ± 41.2 | 35.5 ± 24.7 | 35.1 ± 7.6 |
| T2 | *113.8 ± 39.6 | *152.6 ± 122.6 | *89.6 ± 42.5 | *70.1 ± 35.6 | *80 ± 51.5 | *85.8 ± 29.8 |
| T3 | 15 ± 4.42 | 16.9 ± 15.8 | 17. 2 ± 17.6 | 0.85 ± 0.08 | 2. 9 ± 0.0 | 0.3 ± 0.0 |
| | | | | 26°C | | |
| M1 | *30.46 ± 12.3 | *2.91 ± 1.5 | 0.51 ± 0.41 | 0.058 ± 0.025 | 0.025 ± 0.026 | n. d. |
| T2 | 4.1 ± 3.2 | 1.42 ± 1.14 | *2.87 ± 2.5 | 0.21 ± 0.23 | n. d. | n. d. |
| T3 | 0.075 ± 0.04 | 0.035 ± 0.003 | 0.0013 ±0.0005 | n. d. | n. d. | n. d. |

The numbers indicate the average of 6 replicates of spore counts (X $10^6$ spores/g); M1 (casaminoacids) T2 (corn steep liquor); T3 (peptone); n.d., not detected; * indicate significant difference at 0.05 level.

drying, differences were not observed for the blastospores produced in anything production media (t calculated = -1.48 for M1, t calculated = -0.34 for T2 and t calculated = -0.76 for T3).

The spore germination is very variable and depends of the spore type and also of the oxygen rate (Feng et al., 1994). In our study, the fungus produced mainly blastospores in the media M1 and T3, meanwhile, in the medium T2, produced submerged conidia, according to its morphology presented; according to this, previous studies demonstrated that, the blastospores may have the highest germination rates of 12 h, faster than that presented by submerged conidia of almost 16 h (Thomas et al., 1987). In the medium T2, we found spores with morphology and size of submerged conidia, but the germination rate was as fast as that shown by the blastospores produced in the media M1 and T3. Microcycle phenomenon was observed in all the liquid media that we used.

We called the spores produced in the medium T2 like “blastoconidia” because these share characteristics of both types of spores; the morphology presented was like submerged conidia and the germination rate like blastospore, however, more studies need to be made in order to determine which ingredient of the medium T2 can contribute to the characteristics presented here.

## Storage

The counts of formulations stored at 4 and 26°C are as shown in Table 2. The counts of viable blastospores/ blastoconidia remained higher for formulations maintained at 4 than at 26°C. At this temperature, the blastospores produced in the medium M1 (casaminoacids) were detected after 5 months of storage. The blastospores produced in the media T2 and T3 after 4 months of storage were not detected.

For storage at 4°C, the counts of viable blastospores/ blastoconidia produced in the medium T2 were significantly higher than the counts of blastospores produced in the other media M1 and T3, for one month ($F = 30.6085$, $df = 2, 10$, $P \leq 0.05$), two ($F = 4.4493$, $df = 2, 10$, $P \leq 0.05$) and three months ($F = 5.2286$, $df = 2, 10$, $P \leq 0.05$) after storage; meanwhile, for four months of storage, the counts of blastospores/blastoconidia produced in the media M1 and T2 were similar, without significant difference between both, whereas the blastospores produced in medium T3 were significantly reduced ($F = 15.6381$, $df = 2, 10$, $P \leq 0.05$). For five months of storage, the blastoconidia produced in the medium T2 also showed values significantly higher than the ones presented by the blastospores produced in the other media ($F = 13.8278$, $df = 2, 10$, $P \leq 0.05$). Finally, at six months of storage, the blastoconidia produced in the medium T2 showed significantly higher viability than the blastospores produced in the other media ($F = 46.1144$, $df = 2, 10$, $P \leq 0.05$).

For culture growing, the carbohydrate storage was greatest with glucose as the carbohydrate that supplements the function of storage carbohydrate to supply the organism with an available carbon source under starvation conditions and in some fungi, to supply the spore with an utilizable carbon source (Hegedus et al., 1990). The blastospore production studies using various nitrogen sources at different concentration shows support to production of desiccation-tolerant blastospores (Jackson et al., 1997), whose studies demonstrated that, many complex nitrogen sources supported the production of excellent blastopore yields, but that only casein acid-hydrolyzed and yeast extract produced desiccation-tolerant blastospores. Nutritional compounds found in complex nitrogen sources may be important for regulating desiccation tolerance (Zabriskie et al., 1999). The carbohydrate supplementation in a commercial growth medium, the resultant anabolism and storage of fungal carbohydrates may prolong the shelf life of a *B. bassiana* formulation or increase fungal survival during field

**Table 3.** Bioactivity shown by GHA *B. bassiana* formulations after storage by six months at 4°C against *P. xylostella* third-instar larvae.

| Medium | Accumulated mortality (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1* | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Control 1 (water + Tween 80) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Control 2 (water + Tween 80 + DE) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Blastospores in M1 | 0 | 0 | 0 | 20 | 50 | 70 | 80 | 80 |
| Blastoconidia in T2 | 0 | 0 | 0 | 30 | 70 | 90 | 90 | 90 |
| Blastospores in T3 | 0 | 0 | 0 | 10 | 40 | 60 | 80 | 80 |
| Control + (aerial conidia) | 0 | 0 | 10 | 70 | 80 | 90 | 90 | 90 |

*Days post-application. DE: Diatomaceous earth. The numbers are the average of three replicates.

application (Hegedus et al., 1990).

## Bioassay

The results found for toxic activity from GHA *B. bassiana* formulations maintained at 4°C during six months and tested against *P. xylostella* third-instar larvae are showed in Table 3, where is observed that the untreated controls did not present any effect on the mortality of the larvae, whereas the treatments including the blastospores produced in the three media showed mortality until the three to four days after application, with variable percenttages, at similar way for the positive control (containing aerial conidia); the treatment containing blastoconidia produced in the medium T2 presented the highest mortality percentage, however, this was not higher than the positive control. The treatment containing blastospores produced in the medium T3 presented less mortality.

The mortality percentages were increasing to day 8 when the test was discontinued. The treatment containing blastoconidia produced in the medium T2 presented the highest mortality. This mortality was similar to that of the positive control (90%). The rest of treatments showed also, high mortality percentages without significant difference between them.

These results showed that, the blastospores/ blastoconidia conserved its viability and toxic activity after storage at 4°C and can to be used after some months of storage. More experiments need to be performed in order to determinate the $LC_{50}$ and $LT_{50}$ on *P. xylostella* third-instar larvae.

## Conclusions

The medium containing corn steep liquor produced predominately submerged conidia; meanwhile, the other media produced blastospores. The blastospores produced in the medium containing casaminoacids presented faster germination rates than the other media tested. The spores produced in the medium with corn steep liquor were not affected after storage at 4°C and finally, the blastospores produced in the medium with casaminoacids showed the highest viability at 26°C. The blastospores maintained for six months at 4°C showed high mortality against third-instar *P. xylostella* larvae

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