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Compatibility of polymers to fungi *Beauveria bassiana* and *Metarhizium anisopliae* and their formulated products stability

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ABSTRACT. Stability during storage is a limiting factor in the development and use of bioinsecticides. This study aims to evaluate the compatibility of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* with components used in encapsulated formulations and to evaluate shelf-life in formulations with *B. bassiana*. Sodium alginate and maltodextrin, hemicellulose, and dimethyl sulfoxide solvents were evaluated. Compatibility was evaluated by mixing the components with PDA, and the parameters evaluated were vegetative growth, sporulation and viability. The formulations of fungi were prepared in various concentrations of sodium alginate and then added drop-wise to calcium chloride. To evaluate the stability, formulations were stored at 26.5, 4.0, and -20.0°C. Monthly, samples were taken, and the capsules were placed on Petri dishes with PDA to verify the growth after seven days of incubation. These evaluations were performed by the 12th month. Biopolymers evaluated were compatible with both fungi, exception maltodextrin at 1.5%, which was classified as moderately toxic to *B. bassiana*. Dimethyl sulfoxide was classified as moderately toxic to *B. bassiana* at 3.0% and to *M. anisopliae* at 2.0% and 3.0%. The formulations were stable throughout the 12 months in the conditions evaluated, while for the pure conidia of the fungus, 46% viability in the 6th month at 26.5°C was observed.

Keywords: microbial control, formulations, entomopathogens, encapsulation, shelf-life, microbiology.

Compatibilidade de polímeros aos fungos *Beauveria bassiana* e *Metarhizium anisopliae* e a estabilidade dos seus produtos formulados

RESUMO. A estabilidade no armazenamento é um fator limitante ao desenvolvimento e uso de bioinseticidas. Este estudo objetivou avaliar a compatibilidade dos fungos entomopatogênicos *M. anisopliae* e *B. bassiana* com componentes utilizados em formulações encapsuladas e avaliar a estabilidade de formulações contendo *B. bassiana bassiana*. Alginato de sódio, maltodextrina, hemicelulose e o solvente dimetilsulfóxido foram avaliados. A compatibilidade foi avaliada misturando os componentes ao BDA e os parâmetros para avaliação foram crescimento vegetativo, esporulação e viabilidade. As formulações fúngicas foram preparadas em concentrações de alginato de sódio gotejadas em cloreto de cálcio. Para avaliar a estabilidade, as formulações foram armazenadas em 26,5; 4,0 e -20,0°C. Mensalmente, amostras foram sendo retiradas e as cápsulas colocadas em placas de Petri com BDA para verificar o crescimento após 7 dias de incubação. Essas avaliações ocorreram durante 12 meses. Os biopolímeros avaliados foram compatíveis a ambos os fungos, com exceção de maltodextrina a 1,5%, classificado como moderadamente tóxico para *B. bassiana*. O dimetilsulfóxido foi classificado como moderadamente tóxico para *B. bassiana* a 3,0%; e para *M. anisopliae* a 2,0 e 3,0%. As formulações permaneceram estáveis durante os 12 meses nas condições avaliadas, enquanto para o conídio puro, ao sexto mês já apresentava 46% de viabilidade a 26,5°C.

Palavras-chave: controle microbiano, formulações, entomopatógenos, encapsulamento, teste de prateleira, microbiologia.

Introduction

There are diverse industrial applications for encapsulated products in agribusiness, such as in the production of herbicides and insecticides, not only of chemical origin but also of a biological nature. In the case of biopesticides, microencapsulation could maintain the microbial viability and virulence of infection of the target pest while minimizing or eliminating environmental factors, such as inactivation due to the effects of light and heat (Hanafi, Eltaib, & Ahmad, 2000).

In the encapsulated formulation of a biopesticide, it is of fundamental importance that the constituents, such as the polymer, are compatible with the microorganism to be protected. To maintain the favorable environmental characteristics of the biopesticides, it is fundamental that the constituents of the formulation also are compatible. Gelatin, chitosan,

sodium alginate, cyclodextrins, aliphatic polyesters, such as homo and copolymers of lactate and glycolate (PLA, PGA, and PLGA), poly and caprolactone (PCL) and the polyhydroxyalkanoates, known as PHA, cellulose and cassava starch, can be used as formulation components, among others (Kumari, Yadav, & Yadav, 2010). The biodegradation of these polymers may occur in a biological system through the relaxation of the polymer chain, the breaking of the monomeric unit located at the end of the chain (erosion) or even by the random split of a link at any point along the polymer chain (degradation) (Ré & Rodrigues, 2006; Nobes & Marchessault, 1999).

Conidia that are micro-encapsulated by polymers can be released after the process of polymer relaxation with the hydration of the polymer or after the polymer suffers degradation processes through reaction with oxygen, light, ambient temperature, and especially enzymatic degradation of microorganisms present in the soil (Mohan & Srivastava, 2010).

In Brazil, the use of formulated biopesticides is small because the greatest volume of fungus sold is not formulated and is still in the form it is produced, rice + fungus. This sales strategy demands efficient logistics including refrigerated transport to maintain the quality of the product, cold chambers for storage and conservation, and washing the product when it is used via spraying. With a proper formulation, the volume transported could be reduced, the locations for storage could be smaller with no need for refrigeration, and the application would also be easier.

To obtain a bioinsecticide utilizing entomopathogenic fungi, the process begins with the production of the pathogen, followed by the addition of products that aim to maintain viability and greater efficiency in reaching the target and acting on the insect. A bioinsecticide formulation must enable the maintenance of the stability and viability of the microorganism in storage, increase the ability of the pathogen to reach the target, to preserve the microorganism in the target or substrate and thus increase the control efficacy (Burges, 1998; Batista Filho, Alves, Augusto, & Alves, 1997; Almeida, Batista Filho, Alves, Leite, & Neves, 2008). The use of formulations can also reduce the differences between the efficiency results of microbial control agents in laboratory tests and in the field since they must present the following characteristics: a) good persistence in the plant or body treated and b) good adhesion in the integument of the pest (insect or mite); and in the case of fungus: c) prolong the viability of the entomopathogen in field conditions and d) make the pathogen available at the moment of the susceptible phase of the pest (Almeida, Batista Filho, Alves, Leite, & Neves, 2008).

The development of formulations of bioinsecticides is not so simple. Generally, it works with live organisms, with the intention of keeping them viable for application in the field and control of the target pests. Furthermore, they have specific limitations, in accordance with their nature, such as sensitivity to heat, incompatibility with the chemical agents that compose the formulations, the need for suitable conditions for germination, attractiveness, suitability to the system available on the market, economy, and safety, among others (Almeida, Batista Filho, Alves, Leite, & Neves, 2008).

Microencapsulation is a new technology that acts as a tool for the protection and modulation of the release of substance, and it has been successfully applied to protect substances sensitive to temperature, photo-degradation, oxidization, moisture and other undesirable reactions, giving micro-encapsulated products a greater potential for use (Gonsalves, Costa, Sousa, Cavalcanti, & Nunes, 2009) and providing a good alternative for the protection of the entomopathogens. The authors Carneiro and Gomes (1997). Tamez-Guerra, Mdguire, Behle, Hamm, Sumner, and Shasha (2000) Horaczek and Viernstein (2004) Arthurs, Lacey, and Behle (2006) and Liu and Liu (2009a and b) made use of this technology to formulate biocontrol agents.

Given the above and the realization of how important and advantageous is it to formulate a bioinsecticide, the objectives of this study were to evaluate the compatibility of components and polymers on the fungi *B. bassiana* and *M. anisopliae* used in formulations, to prepare an encapsulated formulation of the fungus *B. bassiana,* and to evaluate the stability of the formulation containing *B. bassiana.*

Material and methods

Fungi

Beauveria bassiana isolate IBCB 66 was obtained from the coffee borer beetle (*Hypothenemus hampei*) in the city of São José do Rio Pardo, São Paulo State, Brazil, and *Metarhizium anisopliae sensu lato* isolate IBCB 425 was obtained from soil from the Atlantic Forest in the city of Iporanga, São Paulo State, Brazil. Both fungi were deposited in the Collection of Entomopathogenic Microorganisms "Oldemar Cardim Abreu" at the Experimental Center of the Biological Institute in Campinas, São Paulo State, Brazil. The pure conidia of

the fungi were stored in Eppendorf bottles, kept at –12°C and multiplied by subculturing in Petri dishes with PDA (Potato-Dextrose-Agar) and then incubated for 12 days at 25.5 ± 0.5°C in B.O.D. (Biochemical Oxygen Demand) to obtain the young cultures used in the compatibility tests of the products.

The pure *B. bassiana* and *M. anisopliae* conidia used in the formulations were supplied by the companies Biocontrol (Sertãozinho, São Paulo State, Brazil) and Toyobo do Brasil Ltda. (Salto, São Paulo State, Brazil), respectively.

Compatibility tests between fungi and biopolymers

Hemicellulose

To evaluate compatibility, the fungi *B. bassiana and M. anisopliae* were cultured in PDA culture media containing 0.1, 0.5, 1.0, and 2.0% (m/v) hemicellulose. The culture media was previously autoclaved at 1.0 atm and 121°C for 20 minutes, with the biopolymer incorporated into the culture media before solidification, at a temperature of approximately 45°C. Then, the mixture was poured into Petri dishes of 9.0 cm diameter, and the control only contained PDA culture media. After the solidification of the media, the fungi were inoculated at three specific points of the culture media with the aid of a platinum loop. The cultured dishes were incubated in a germination chamber for 7 days at a temperature of 25.5 ± 0.5°C and a photoperiod of 12 hours. Evaluations of the vegetative growth were performed by the direct measurement of the microbial growth halo in two perpendicular directions. Then, these colonies were cut out using a flame-sterilized scalpel and placed in test tubes with a 10 mL autoclaved suspension of distilled water and spreader-sticker (Tween®80) at 0.1% (m/v). This suspension was homogenized in a tube stirrer for extraction and subsequent determination of the number of conidia in a Neubauer chamber.

The evaluation of the viability was performed in Petri dishes in which a thin layer of PDA was placed with pentabiotic (0.5 g L^{-1}) to avoid bacterial contamination. After solidification of the medium, each dish received 0.1 mL of fungal suspension, prepared for the conidia count. The suspensions were spread with a Drigalski spatula, previously flamed and properly cooled.

Then, the dishes were incubated for 24h in B.O.D. at 25.5 ± 0.5°C with a photoperiod of 12 hours. After this period, the dishes were observed under a microscope (Leica, model DM 500), with 400x magnification to count 100 conidia, germinated and not-germinated, thereby establishing the proportion germinated.

Each treatment was evaluated with six repetitions for radial growth, sporulation and viability.

The calculation of the compatibility factor for the products analyzed used the Biological Index (BI) proposed by Rossi-Zalaf, Alves, Lopes, Neto and Tanzini (2008):

$$BI = \frac{47[VG] + 43[SP] + 10[GER]}{100}$$

where: BI = is the Biological Index, VG = is the vegetative growth percentage after seven days compared to the control, SP = is the sporulation percentage of the colonies after seven days compared to the control, and GER = is the germination percentage of the conidia after 24 hours.

The values of BI for classification of the product are as follows: 0–41 = toxic (T); 42–66 = moderately toxic (MD), and > 66 = compatible (C). Dimethyl Sulfoxide (DMSO), sodium alginate and maltodextrin.

DMSO solvent was added to the culture medium in concentrations of 0.5, 1.0, 2.0, and 3.0% (m/v), and 0.5, 1.0, and 1.5% (m/v) sodium alginate and maltodextrin were also added. The test followed the same methodology described in the previous section.

Formulations of capsules with sodium alginate (Patent number 2015 016 269 BR10 3)

Capsules loaded with conidia were prepared according to Pasqualim et al. (2010), with modifications. $CaCl_2$ was prepared briefly in a 1.0 L beaker at concentrations of 0.01, 0.03, and 0.05 M. In a second beaker, 1.0% (m/v) sodium alginate was initially prepared, with the subsequent incorporation of 0.5% (m/v) of the fungi *M. anisopliae* or *B. bassiana* and 0.02% (m/v) Tween 80®. The suspension was homogenized by turbo extraction (Ultra-turrax, IKA T25), and the pH was measured (pH meter Metrohm model 827).

Under agitation, the sodium alginate solution and the fungus were slowly added to the $CaCl_2$, forming the capsules. The phase transfer was performed with a peristaltic pump (Watson-Marlow Inc., TPM 600 55RPM) at a flow of 35 drops $min.^{-1}$

Once the capsules were formed, they were placed in sieves and washed with distilled water to remove the excess $CaCl_2$. The capsules were places in an oven to dry at 24.0°C for 48h.

The conidia were counted after the solubilization of the capsules in PBS buffer, which was prepared by dissolving 16.0 g of NaCl, 0.4 g of KCl, 2.88 g of $Na_2HPO_4 \cdot 2H_20$, 0.48 g of KH_2PO_4 and in 1000 mL of ultrapure water.

Stability test

The dried capsules with 1.0% (m/v) *B. bassiana* were formulated in 0.7%, 1.0% or 1.3% (m/v) sodium alginate and stored for 12 months at three different temperatures: a) 26.5°C, b) 4.0°C or c) –20.0°C (Table 1). The samples were evaluated monthly for stability. The encapsulated samples were placed in Petri dishes with PDA, and the vegetative growth of the fungus was monitored until the seventh day. In addition to the growth, samples were observed for morphological changes of the fungus during the growth of the colony. The pure conidia of the fungi were also evaluated.

Table 1. Formulations encapsulated in sodium alginate by dripping in $CaCl_2$.

Formulation code	Sodium alginate (%)	*Beauveria bassiana* (%)	$CaCl_2$ solution	Storage temperature (°C)
A	1.0	1.0	0.03 M	26.5
B	1.0	1.0	0.03 M	4.0
C	1.0	1.0	0.03 M	-20.0
4	0.7	1.0	0.03 M	26.5
5	0.7	1.0	0.03 M	4.0
6	0.7	1.0	0.03 M	-20.0
7	1.3	1.0	0.03 M	26.5
8	1.3	1.0	0.03 M	4.0
9	1.3	1.0	0.03 M	-20.0

Statistical analysis

The SISVAR program (Version 5.3) was used for statistical analysis. The trials conducted were completely randomized. Each test was completed six times, and the data were subject to variance analysis and significance testing, where the Tukey test was performed at 5% to compare the averages.

Results and discussion

Compatibility tests of the biopolymers with the fungi

For the fungus *M. anisopliae*, it was observed that for the vegetative growth and sporulation parameters, there was no significant difference compared to the control at the concentrations evaluated, with the exception of 1.0% hemicellulose (Table 2).

The evaluation of the viability was statistical different compared to the control and to the 2.0% (m/v) hemicellulose treatment, whose viability values were 100 and 97.6%, respectively (Table 2).

The quantities of the biopolymers sodium alginate and maltodextrin evaluated were statistically equal to the control in relation to the vegetative growth, sporulation and viability parameters (Table 2).

Table 2. Averages of vegetative growth, sporulation and conidia viability of the IBCB 425 isolate of *Metarhizium anisopliae* cultured in the presence of polymers and solvent after a seven-day incubation at 25.5 ± 0.5°C with a photoperiod of 12 hours.

ent	Vegetative growth (cm)	Sporulation ($x10^7$)[1]	Germination (%)
Control	2.09 ± 0.15 b	3.35 ± 0.62 b	100.0 ± 0.00 a
0.1% hemicellulose	2.25 ± 0.15 b	6.20 ± 1.05 ab	98.6 ± 0.56 ab
0.5% hemicellulose	2.45 ± 0.10 ab	5.40 ± 2.42 ab	98.6 ± 0.60 ab
1.0% hemicellulose	2.81 ± 0.22 a	7.25 ± 1.14 a	99.0 ± 0.30 ab
2.0% hemicellulose	2.48 ± 0.22 ab	5.35 ± 0.42 ab	97.6 ± 0.63 b
F test	4.83★	4.08★	4.25★
Control	2.24 ± 0.11 a	4.99 ± 2.13 a	97.8 ± 0.22 a
0.5% DMSO[2]	1.77 ± 0.10 a	3.68 ± 2.27 ab	92.8 ± 0.33 ab
1.0% DMSO	2.07 ± 0.08 a	2.18 ± 1.58 bc	86.4 ± 0.51 b
2.0% DMSO	1.77 ± 0.19 a	1.46 ± 1.62 c	84.0 ± 0.34 b
3.0% DMSO	1.78 ± 0.06 a	0.71 ± 0.02 c	92.4 ± 0.00 ab
F test	2.932[ns]	12.73★	4.478★
Control	2.11 ± 0.13 a	8.38 ± 1.21 a	97.4 ± 0.56 a
0.5% sodium alginate	2.22 ± 0.16 a	8.60 ± 1.50 a	97.0 ± 0.71 a
1.0% sodium alginate	2.76 ± 0.16 a	9.58 ± 1.23 a	95.6 ± 0.51 a
1.5% sodium alginate	2.60 ± 0.11 a	6.58 ± 0.91 a	96.2 ± 0.40 a
0.5% maltodextrin	2.44 ± 0.19 a	6.10 ± 1.51 a	95.2 ± 0.76 a
1.0% maltodextrin	2.12 ± 0.14 a	6.30 ± 2.45 a	97.0 ± 0.76 a
1.5% maltodextrin	2.63 ± 0.23 a	6.10 ± 1.26 a	97.2 ± 0.42 a
F test	3.09[ns]	6.16[ns]	2.19[ns]

[1]Data processed at $\sqrt{x} + 0.5$ in the analysis, but the original data are in the table; [2]DMSO = dimethyl sulfoxide; [ns] = not significant; ★ significant at 5% probability by Tukey's test.

The compatibility study with the DMSO solvent demonstrated no significant difference between the treatments for the vegetative growth parameter. However, the sporulation and the viability were decreased due to the solvent in the culture media. For sporulation, only the concentration of 0.5% DMSO was statistically equal to the control, and in the case of viability, 1.0% and 2.0% differed from the control (Table 2).

With respect to the fungus *B. bassiana*, the hemicellulose concentrations evaluated were statistically equal to the control in the three parameters evaluated (Table 3). Investigating the use of DMSO solvent, it was shown that vegetative growth in the 2.0% and 3.0% DMSO groups differed statistically from the control. The sporulation evaluated demonstrated a lower number of conidia at all concentrations of DMSO and were significantly different from the control. In the viability parameter, there was no significant difference between the treatments.

The vegetative growth of the fungus was not affected by any of the sodium alginate concentrations evaluated, and the treatment groups even presented higher growth values than the control. The maltodextrin treatments did not differ statistically from the control. There were effects on sporulation

when 1.5% sodium alginate was used, which resulted in a higher number of conidia than the control. For maltodextrin, the 1.0% and 1.5% concentrations did not differ statistically from the control. For the viability parameter, all of the concentrations evaluated for both of the polymers were statistically equal to the control.

Table 3. Averages of vegetative growth, sporulation and conidia viability of the IBCB 66 isolate of *Beauveria bassiana* cultured in the presence of polymers and solvent after a seven-day incubation at 25.5 ± 0.5°C with a photoperiod of 12 hours.

Treatment	Vegetative growth (cm)	Sporulation ($x10^7$)[1]	Germination (%)
Control	1.86 ± 0.14 a	0.65 ± 7.63 a	99.6 ± 0.68 a
0.1% hemicellulose	1.68 ± 0.09 a	0.51 ± 2.27 a	100.0 ± 0.0 a
0.5% hemicellulose	1.43 ± 0.08 a	0.65 ± 0.93 a	99.2 ± 0.42 a
1.0% hemicellulose	1.82 ± 0.07 a	0.65 ± 0.85 a	99.8 ± 0.18 a
2.0% hemicellulose	1.81 ± 0.12 a	0.54 ± 0.84 a	99.2 ± 0.49 a
F test	5.07[ns]	0.410[ns]	0.821[ns]
Control	1.66 ± 0.11 a	0.13 ± 2.13 a	99.6 ± 0.22 a
0.5% DMSO[2]	1.60 ± 0.10 ab	0.67 ± 2.27 b	99.4 ± 0.33 a
1.0% DMSO	1.77 ± 0.08 a	0.72 ± 1.58 bc	99.2 ± 0.51 a
2.0% DMSO	1.37 ± 0.19 b	0.66 ± 1.62 b	99.6 ± 0.34 a
3.0% DMSO	1.32 ± 0.06 b	0.17 ± 0.02 c	100.0 ± 0.0 a
F test	7.946⋆	15.847⋆	0.611[ns]
Control	2.06 ± 0.11 b	0.16 ± 0.16 bc	97.0 ± 0.83 a
0.5% sodium alginate	2.58 ± 0.27 ab	0.16 ± 0.32 bc	97.4 ± 0.51 a
1.0% sodium alginate	3.16 ± 0.22 a	0.33 ± 1.95 ab	97.6 ± 0.60 a
1.5% sodium alginate	2.45 ± 0.31 ab	0.52 ± 2.01 a	97.0 ± 0.66 a
0.5% maltodextrin	1.99 ± 0.15 b	0.41 ± 1.31 a	99.0 ± 0.47 a
1.0% maltodextrin	2.03 ± 0.22 b	0.77 ± 1.37 c	97.8 ± 0.84 a
1.5% maltodextrina	1.76 ± 0.10 b	0.50 ± 0.46 c	97.8 ± 0.30 a
F test	6.743⋆	24.204⋆	0.982[ns]

[1]Data processed at $\sqrt{x} + 0.5$ in the analysis, but the original data are in the table; [2]DMSO = dimethyl sulfoxide; [ns] = not significant; ⋆ significant at 5% probability by Tukey's test.

This is, to the best of our knowledge, the first study evaluating polymers. This study is important because it contributes to the knowledge and development of new formulations. One of the items indispensable to the manufacture of a formulation is to verify that the components to be added are compatible with the fungi that will be added to the formulation.

With respect to the classification by BI, the majority of the polymers in the concentrations evaluated were considered compatible with the fungi. The DMSO solvent, at a concentration of 3.0% (m/v) (for *B. bassiana*) and at concentrations of 2.0% and 3.0% (m/v) (for *M. anisopliae*) was classified as MT. The polymer maltodextrin, at a concentration of 1.5% for the fungus *B. bassiana*, also received this classification. The BI values and the classification of the compatibility of the fungi *M. anisopliae* and *B. bassiana* is presented in Table 4.

Most of the concentrations used were compatible with the entomopathogenic fungi evaluated, leaving a good safety margin to work with different products that could make up the formulation.

Table 4. BI and classification of the polymers and solvent for the compatibility of the fungi *Beauveria bassiana* (IBCB 66) and *Metarhizium anisopliae* (IBCB 425) in the laboratory.

Treatment	BI *B. bassiana*	Classification⋆	BI *M. anisopliae*	Classification⋆
0.1% HC[1]	89.97	C	139.6	C
0.5% HC	93.87	C	134.1	C
1.0% HC	103.7	C	165.9	C
2.0% HC	91.49	C	134.2	C
0.5% DMSO[2]	76.24	C	78.33	C
1.0% DMSO	82.59	C	71.04	C
2.0% DMSO	69.59	C	58.32	MT
3.0% DMSO	52.88	MT	52.95	MT
0.5% AS[3]	89.25	C	115.1	C
1.0% AS	169.45	C	120.0	C
1.5% AS	201.41	C	101.4	C
0.5% MD[4]	163.0	C	95.08	C
1.0% MD	76.45	C	89.46	C
1.5% MD	63.26	MT	99.84	C

⋆0 to 41 - toxic (T); 42 to 66 - MT; > 66 compatible (C), 1HC = hemicellulose; 2DMSO = dimethyl sulfoxide; 3AS = sodium alginate; 4MD = maltodextrin.

Formulations of capsules with sodium alginate

This encapsulation method was a simple, fast, efficient and high performance process; the components used were compatible with the fungi evaluated. Some characteristics can also be observed, such as the size of the capsules, which is related to the size of the drop that falls into the $CaCl_2$ solution and the transfer speed of the solution containing the conidia and the polymer. It was also observed that the greater the molarity of the calcium chloride solution, the more spherical the capsules that were formed. When 0.01 M $CaCl_2$ was used, the capsules demonstrated a more elongated shape, different from the spheres formed in the 0.03 and 0.05 M $CaCl_2$. The alginate is an anionic linear polymer composed of β-D-mannuronic and α-L-guluronic acids, which characteristically form a gel when in contact with the calcium ion solution by cross linking between the carboxylate anions of the guluronate of the alginate and the calcium ions (Liu & Liu, 2009a). This polymer network is responsible for surrounding and encapsulating the conidia.

Once the particles are obtained, the capsules were dried to reduce or eliminate the presence of moisture, to inhibit the germination of the conidia and the growth of contaminant microorganisms. Furthermore, when stored at low temperatures, the wet capsules could freeze, making the conidia unviable. In this preparation process, the presence of Tween 80 facilitated the homogeneous dispersion of the fungus mixture and polymer.

Table 5 has some of the parameters evaluated in the formulations composed of 1.0% sodium alginate (m/v), 0.02% Tween 80, 0.5% fungus (m/v) and 0.05 M $CaCl_2$. The pH values measured were between 6.99 and 7.39, which was a similar result to that observed by Liu & Liu (2009a) where the authors found that the pH value of the suspensions varied from 6.2 to 6.7.

Table 5. Data from the formulations of the fungi *Beauveria bassiana* and *Metarhizium anisopliae* encapsulated in sodium alginate by drop-wise addition to 0.05 M $CaCl_2$.

	M. anisopliae	*B. bassiana*
Fungal concentration before encapsulation	2.2×10^8 con mL^{-1}	9.5×10^8 con mL^{-1}
Fungal concentration after solubilization in PBS	2.3×10^7 con mL^{-1}	2.6×10^8 con mL^{-1}
Average yield (g) (%)	1.46 (97.7%)	1.57 (99.0%)
Viability of the fungus (%) after solubilization in PBS	90.0%	99.0%
pH of the suspension	7.39	6.99

Another important observation of the formulations was that the performance was very high (Table 5), which indicated minimal losses during the process. In addition, the solubilization in the PBS buffer did not impact the viability and germination of the conidia of the fungi.

The selection of the encapsulating agency depended on the method used to form the capsules, the type of application of the product in the field and even on the action mechanism. The release of the active substance, in this case conidia, can occur through mechanical stimulation (breaking of the microcapsules by pressure), by moisture via the swelling process or by temperature or pH variation in the medium containing the microcapsules (Ré & Rodrigues, 2006).

The biodegradable polymers require degradation via the action of naturally occurring microorganisms, such as bacteria, fungi and algae, and they can be consumed in weeks or months under conditions favorable to biodegradation. Those that have attracted the most attention are those obtained from renewable sources due to the lower environmental impact regarding the origin and the positive balance of carbon dioxide (CO_2) after composting, among other impacts (Brito, Agrawal, Araújo, & Neto, 2011).

Stability test

The stability of the formulations was evaluated in different storage conditions. The most significant result was obtained at an ambient temperature. In months 1–6, a 47% decrease in the viability of the pure conidia of the fungus was observed, reducing from 97% to 46%. For formulations evaluated in the same conditions, there was no decrease in viability, indicating that each capsule placed in a dish generated a fungal colony. Under storage conditions in a refrigerator or freezer, for up to 12 months, no significant decrease in the viability of the pure conidia was observed (Figure 1).

Carneiro and Gomes (1997) evaluated encapsulated formulations containing a mixture of sodium alginate (1.0%), clay (5.0%), streptomycin sulfate (1.0%) and a suspension of conidia, 1.0×10^9 conidia mL^{-1} to *Paecilomyces lilacinus*. The dry pellets were stored in two different conditions (ambient at 18°C to 25°C and refrigerated at 7°C) and evaluated monthly for a period of 12 months. The authors found that until the 5th month of evaluation, at ambient temperature, the germination index stayed between 95% and 100%, and in the following seven months, the viability gradually decreased, showing within 12 months a germination percentage of 20%. As for the product stored under refrigeration, the germination remained stable throughout the evaluation period, with 95% to 100% germination. This result differs from that found in this study because the formulations encapsulated in sodium alginate remained viable, even at ambient temperature, for 12 months.

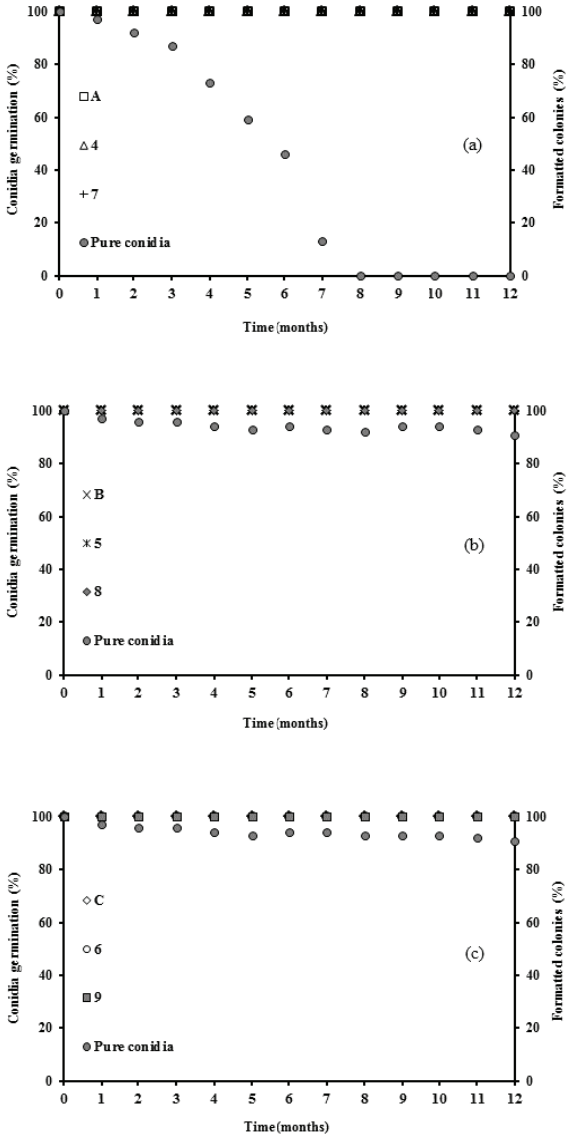

Figure 1. Germination of the pure conidia and the fungal growth of the encapsulated formulations of the fungus *B. bassiana* at different storage temperatures, where (a) 26.5°C, (b) 4.0°C and (c) -20.0°C.

Liu and Liu (2009a) investigated the formulations of *M. anisopliae* encapsulated in sodium alginate. The stability of these formulations was evaluated for 6 months at a temperature of 4°C. The encapsulated conidia remained viable up to the 6th month, with 80% viability in this period. For the pure conidia, the germination rates demonstrated significant declines over the months, reaching 50% in the 6th month, corroborating the results found for *B. bassiana* in this study.

This germination versus temperature evaluation is extremely important because positive results could enable large-scale production, storage, marketing and distribution of this biological control agent without the risks of loss of efficiency. These characteristics are also favorable with respect to attracting companies that invest in microorganisms products (Kerry, 1990).

The encapsulation of the fungus in sodium alginate makes the application and distribution in soils easier, as well as protecting the conidia from external action. The resistance provided by the granules also enables use in aquatic environments without the destruction of the granules (Pethkar & Paknikar, 2003).

Therefore, one of the objectives of formulating a product was achieved. The product remained viable for a year in the three conditions evaluated. This is a major advance, particularly because currently available formulations last up to 90 days in non-refrigerated conditions, and this tested product did not lose viability in 12 months. A suitable shelf-life for a mycopesticide product at an ambient temperature is an essential requirement for acceptance and commercialization (Jones & Burges, 1998). Another advantage is the reduction in transport volume; the quantity formulated is much smaller than when using the traditional rice + fungus. In addition, the logistics of the transport are easier, and the cost is reduced since refrigerated trucks are unnecessary. All of these advantages will make this product cost-effective, attractive and viable to the consumer, as well as environmentally friendly.

Conclusion

The biodegradable polymers hemicellulose, sodium alginate and maltodextrin were compatible with the fungi *B. bassiana* and *M. anisopliae sensu lato*. The technique of drop-wise addition to calcium chloride demonstrated high performance, high encapsulation efficiency and perfect compatibility with the microorganisms. The conidia encapsulated in sodium alginate were stable for 12 months of storage in the conditions evaluated (ambient, refrigerator, and freezer).

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