Effects of the Temperature and Storage on Formulations with Mycelia of B*eauveria bassiana* (Bals.) Vuill and *Metarhizium anisopliae* (Metschn.) Sorok.

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ABSTRACT

This research deals with preparation of experimental formulations with mycelia of Beauveria bassiana *and* Metarhizium anisopliae*. The formulations were prepared with sodium alginate and pre-gelatinized corn-starch. They were kept at 25, 30 and 35±1°C. Pre-gelatinized corn starch was more suitable that the sodium alginate for the preparations of formulations with mycelia of* B. bassiana *and* M. anisopliae.

Key words: Formulation, Entomopathogenic fungi, Microbial Control.

INTRODUCTION

The entomopathogenic fungi are pest control agents of economic importance used as in several countries, including Brazil. These biological insect control agents are distinguished by their abundance of genera and species in the control of pest. (Veen, 1968; Alves, 1986; Marques & Alves, 1996; Roberts, 1997).

In Brazil, as in other regions of the world, *M. anisopliae* and *B. bassiana* species are being the most studied and used due to their wide range fungal activity. From 1970 to 1991, approximately 38,000Kg of conidia of *M. anisopliae* were produced in the state of Pernambuco for spraying 474,000 hectares of *Mahanarva posticata* leafhopper infested sugarcane fields (Marques, 1992). It has also been tested for the control of the *Nasutitermes sp*. termite (Malagodi & Veiga, 1995).

The *B. bassiana* fungus is being used in pest control *Cosmopolites sordidus*, *Cornitermes cumulans* and *Disploschema rotundicolle*. Its potential has also been assessed for the control of other pests (Alves, 1992).

There is little published information available about the formulation of entomopathogenic fungi because the technology is still held as an industrial secret. However, it is known that it is a mixture of several compatible products that include an active ingredient, typically conidia, a thinner and or a disperser, a wetting agent and an adherent (Latgé & Moletta, 1988). More recently, the production of these fungiis being done in the form of dry mycelium, since this could best resist the adverse condition until it comes in the contact with the agent.

McCoy *et al* (1975) produced *Hirsutella thompsonii* mycelium and mentioned that the inclusion of sugarcane syrup in the preparation of the fungi, increased the production of conidia and they recognised the need to develop storage methods which do not require low temperature due to the lack of viability of mycelium stored at 10°C after a ten day period. Abdel-Halim *et al* (1986) studied the encapsulation of conidia of *Penicillium chrysogenum* using 6% of sodium alginate, suspension of conidia and a solution of 0.2M of calcium chloride. When encapsulated in this way, the fungus showed an increase in the production of penicillin. The capsules were destroyed by the growth of the fungus liberating it in the culture.

Cranston (1983) tested the growth of a great number of micro-organisms, including bacteria, fungi and yeast in a medium in which agar was substituted by sodium alginate. The average result was similar to the corresponding medium.

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Fravel *et al* (1985), studied the encapsulation of five fungi and one bacteria with a clay-alginate mixture. They were prepared in granules formed with a suspension of 1% of sodium alginate and 10% of clay with calcium chloride. All fungi were viable after the formation of granules. The granules were dried and stored under normal weather conditions for more than three months showing more resistance than the conidia in the calcium gluconate formulations. Rombach *et al* (1986) produced preparations with the *Metarhizium anisopliae* and *Paecilomycenes liliacinus* mycelia using 1.5% of yeast extract and 1.5% of sugarcane syrup. The mycelia were dried and stored at low temperatures until the preparation for the use in the field. The use of mycelial formulations can simplify the mass production of entomopathogenic fungi and these preparations could be efficient in insect control.
Sasha *et al* (1984) studied several starch complexes for the encapsulation of herbicides with the objective of finding a substitute for the alkaline sodium hydroxide being used until then. The aim was reached with the used of pre-gelatinised corn starch. Pereira (1987) and Pereira & Roberts(1991) obtained preparations of dry *M. anisopliae* and *B. bassiana* mycelia and studied the survival and capacity of conidia production from them after mixing with sodium alginate, corn starch and sugars when stored at a temperature of 22^{O}C and 4^{O}C, verifying the evidence of beneficial treatment with sugar. They concluded that these formulations can represent a viable alternative to the use of entomopathogenic fungi in pest control, as well as for their dissemination.

Rombach *et al* (1988) studied the storage of dry mycelium of the Chinese variation *B. bassiana* at -20, 5, 25 and 35^{O}C during a period of one to thirty-two weeks. At 25^{O}C, the number of conidia produced was very lower. At 35^{O}C, there was no viability of mycelium after a week. Thus, they concluded that the dry *B. bassiana* mycelium can only be stored for long periods of time at a temperature of -20^{O}C.

Knudsen *et al* (1990) formulated the *Beauveria bassiana* mycelium in granules of sodium alginate with and without the addition of ground wheat. After five months of storage at room temperature, the fungi with most spore production came from the granules with wheat, with 2.45 x 10^{8} conidia per granule. These, once placed on seedlings of wheat infested with *Schizaphis graminum*, caused the death of three to forty-four percent of aphis, against 0% in the control. Andersch (1992) researching the *M. anisopliae* mycelium (BIO 1020), mentioned that storage with the contact of oxygen had a negative influence on the product. This process being air-tight and stored at 4^{O}C kept its viability and biological activity for twelve months and tests done against *Otiorhynchus sulcatus* proved a 60 to 100% death rate. Krueger *et al* (1992) reported that the death rate of *Rhizotrogus majalis* and *Popillia japonica* larvae occurred more rapidly in soils inoculated with the dry mycelium particles against those with the *M. anisopliae* conidia. However, the total death rate did not differ considerably between the two types of inoculation. Stenzel *et al* (1992) presented a granular formation with the *M. anisopliae* mycelium (DSM 3884) developed for horticulture use. The granules were more effective when mixed with the soil at a proportion of 1.0 g/litre of soil. Research undergone between 1989 and 1991 managed a control of 74 to 81% over ornamental plants pests. Harrison *et al* (1993) studied the effect of seven types of soil, three densities, six mixtures of soil and pH from 5 to 8, on conidia production of *M. anisopliae* (BIO 1020 and MADA) in the mycelial format. The conidia production was higher on soils of vases followed by cupric sand, thin sand, turf and candller sand, being that after one to three weeks, it was similar to autoclaved and natural soil.

In spite of the importance of the said entopathogenic fungi, the lack of formulations restricts their wider use in pest control (Marques, 1992; Alves, 1992; Wraight & Bradley, 1996). Thus, this research aims at the elaboration of formulations with *M. anisopliae* and *B. bassiana* mycelia, trying to identify supporting agents, which could improve the preservation of these propagules at a temperature of 25, 30 and 35^{O}C.

MATERIALS AND METHODS

We used E9 lineages of *M. anisopliae* and 447 of *B. bassiana*. Formulations were prepared by taking 900mL of a medium of potato broth,

dextrose, yeast extract and streptomicin sulphate, containing mycelial mass from each fungi and blended for sixty seconds. The resulting dense liquids were divided into three portions:
a) 400mL to be used with formulations with sodium alginate;
b) 400mL for formulations with pre-gelatinized corn starch;
c) 100mL to be filtered in a Buchner filter, dried in an unit of laminated flow and used as control.

a) Sodium alginate formulations: The flasks containing 400mL of medium plus suspended mycelial mass of each fungus were left for a period of 4h to decant. After this period of time, 100ml of floating material was eliminated. The resulting suspensions. were mixed separately with 4g of sodium alginate, which had been diluted in 8ml of 96% alcohol and later blended for 60 seconds. To this 6g of corn starch was also added in order to add more consistency to the preparation. The mixture of each fungus, was placed on a funnel with a rubber tap and allowed to fall, drop by drop, into 200mL of a sodium chloride solution (4%). (Pereira & Roberts 1991). This resulted granules formation, which were placed on a sieve for drying in a laminar flow chamber for 24 h. Portions with 1.5g were stored in plastic tubes (3.0cm x 2.5cm and 13mL). Three repetitions were made and the tubes were closed and stored in a BOD stove at a 25 ± 1^{o}, 30 ± 1^{o} and 35 ± 1^{o}C. The spore production capacity was assessed labelling samples in BDA plus antibiotics before the storage and after 15, 30, 60, 90 and 120 days.

b) Formulations with pre-gelatinized corn starch: As in the alginate preparations, the flasks containing 400mL of medium and suspended mycelium were decanted. After this, 200mL of floating *B. bassiana* mycelial mass and 100mL of the suspension containing *M. anisopliae* mycelium were eliminated. Then, 25g of pre-gelatinised starch were added to the mycelial suspension and blended for sixty seconds for homogenisation. The mixtures were placed to dry in the laminar flow unit for 24h (Pereira & Roberts 1991).

After drying, 1.5g of the preparation were put in plastic tubes as above. Three repetitions were made and the recipients were taken to the stoves which were set at temperatures of 25 ± 1^{o}, 30 ± 1^{o} and 35 ± 1^{o}C.

As in the alginate formulations, their capacity of spore production were also measured before the storage and after 15, 30, 60, 90 and 120 days placing portions in BDA with antibiotics.

c) Mycelial mass without preparation: After drying, portions with 0.7g of *B. bassiana* mycelium and 0.9g of *M. anisopliae* mycelium were stored in plastic tubes identical to those used for the formulations with alginate and corn starch in three repetitions and kept in the stove at temperatures of 25 ± 1^{o}, 30 ± 1^{o} and 35 ± 1^{o}C. The spore production capacity was also assessed before the storage and after 15, 30, 60, 90 and 120 days.

Experiment with the *B. bassiana* and *M. anisopliae*.

a) Laboratory studies : As it was said earlier, all the formulations together with the pure dried mycelium of both dried fungi were placed in plastic tubes and placed in stoves for BOD at 25, 30 and 35 ± 1^{o}C for 120 days.

The experimental design was entirely rondonized, with three repetitions consisted of the six following treatments:
Trat. 1. Pure *B. bassiana* mycelium;
Trat. 2. *B. bassiana* mycelium with sodium alginate;
Trat. 3. *B. bassiana* mycelium with pre-gelatinised corn starch;
Trat. 4. Pure *anisopliae* mycelium;
Trat. 5. *M. anisopliae* mycelium with sodium alginate;
Trat. 6. *M. anisopliae* mycelium with pre-gelatinized corn starch.

Spore production capacity of the formulations: In order to verify the viability and conidia production of the formulations, some of their particles, measuring approximately $23.75mm^{2}$ were placed on Petri dishes with BDA plus antibiotics and stored to the BOD stove at 25 ± 1^{o}C for a period of 7 days. After this samples were taken (disks 5.5mm of diameter) from A, B and C treatments. The samples were placed in small tubes with sterile water and 0.1% of Tween 80 shaken for 60

seconds. Then, the conidia were counted in a Neubauer chamber with the aid of a microscope of the brand American Optical.

The spore production capacity of the treatments before and after storage was also assessed on the soil. In order to do that, plastic cups with 8cm in diameter and 7cm in height were used with an Eutrophic Nitosol, (pH 5.1). Three samples of the preparation were inoculated, which were covered with glass and stored at 26±1^{o}C for seven days.

b) Field studies : These studies were aimed at assessing the capacity of spore production as well as the loss of particles in the formulations left in the field. Samples of the treatments before the storage and on the 15th, 30th,60th and 90th day, kept at 25±1^{o}C, were taken to the field, placed in the soil at a depth of 1.0cm and at the axis and leaf shelt of 8 months old sugar cane plants. The observations were done at sugar cane plantations at the experimental field , from clay to high clay texture (Kandiudalfic Eutrudox), as determined by Vidal-Torrado & Sparovek (1993).

RESULTS AND DISCUSSION

The data of the spore production from the *B. bassiana* and *M. anisopliae* fungi formulated with sodium alginate and corn starch stored at 25±1^{o}, 30±1^{o} and 35±1^{o}C for 120 days can be seen in Tables 1, 2 and 3. Before the storage, the most productive preparations were those with sodium alginate for both fungi, followed by corn starch for *B. bassiana* (Table 1). At a temperature of 25±1^{o}C, on the 15th day of storage, the formulations were amongst the three most productive and on the 30th day, the *M. anisopliae* formulation with sodium alginate was already non-viable. The same happened with the preparation with dry mycelium on the 60th day. At this temperature, Rombach *et al* (1988), reported that after two weeks, the *B. bassiana* mycelium were non-viable. On the 90th day of storage, the formulations of *B. bassiana* with alginate and starch were the best, followed by dry mycelium and last was the preparation with *M. anisopliae* mycelium plus corn starch. After 120 days, the formulation of *B. bassiana* with corn starch was perfectly viable, followed by the dry mycelium preparation. On the other hand, the amount of spores verified in the formulation of *B. bassiana* with sodium alginate until the 90th day could be partly due to the percentage of corn starch in it. This fact is corroborated by Knudsen et al (1990) who, when working with this formulation, made mixtures with and without wheat flour, obtained similar results to those found by this research. The maximum spores production found in preparations of *B. bassiana* in subsequent dates of storage could be regarded due to the lack of uniformity in the size of the samples placed in the BDA plus antibiotics or by the collection for the counting of conidia.

At a temperature of 30±1°C, as shown in the Table 2, on the 15th day of storage, the dry mycelium preparation and the formulations with *B. bassiana* were more productive than the formulations with *M. anisopliae*. However, the preparation with alginate became non-viable. On the 30th day, the best treatments were the dry mycelium and the preparation with corn starch and the *B. bassiana* fungus. After this period of time, the preparation with *dry M. anisopliae* mycelium was non-viable. On the 60th day, the best formulations were the *B. bassiana* with alginate and corn starch. However, on the 90th day of storage, only this fungus with corn starch presented viable and with the capacity of the production of conidia, going on until the 120th day.

On the 15th day of storage at a temperature of 35±1°C, the formulation with alginate and the treatment dry *M. anisopliae* mycelium were already non-viable. On the other hand, all the treatments with *B. bassiana* continued viable and with total capacity for the porduction of conidia, as can be seen on table three. After 30 days the best treatments were *B. bassiana* formulated with corn starch and dry mycelium.

Table 1. Media number of conidia x 10^7 produced in granules (size 23,75mm^2) of *B. bassiana and M. anisopliae* mycelium *f*ormulations. 25±1^{o}C and 12 h light.

Formulations	AN/A	Days of storage				
		15	30	60	90	120
B. bassiana						
Dry mycelium	9,43bc*	8,27ab	9,40a	15,33ab	7,90bc	1,90b
Sodium alginate	17,00a	10,87ab	3,20ab	18,00a	15,00a	0,00b
Corn starch	12,00abc	13,67a	8,23a	11,00ab	14,33ab	10,90a
M. anisopliae						
Dry mycelium	6,90c	5,80b	8,17a	0,00d	0,00d	0,00b
Sodium alginate	15,00ab	13,33a	0,00b	0,00d	0,00d	0,00b
Corn starch	7,30c	8,00ab	6,70a	5,20cd	5,27cd	0,00b

AN/A - Before storage.

* Means with the same letter are not significantly different according with Tukey test (P < 0.05)

Table 2. Media number of conidia x 10^7 produced in granules (size 23,75mm^2) of *B. bassiana and M. anisopliae* mycelium *f*ormulations. 30±1^{o}C and 12 h light

Formulations	AN/A	Days of storage				
		15	30	60	90	120
B. bassiana						
Dry mycelium	9,43bc*	14,60a	13,33a	8,97b	0,00b	0,00a
Sodium alginate	17,00a	12,00ab	7,07bc	19,67a	0,00b	0,00a
Corn starch	12,00abc	9,60bb	10,27ab	16,00ab	11,67a	0,30a
M. anisopliae						
Dry mycelium	6,90c	4,33bc	0,00c	0,00c	0,00b	0,00a
Sodium alginate	15,00ab	0,00c	0,00c	0,00c	0,00b	0,00a
Corn starch	7,30c	5,53b	2,63c	3,27bc	0,00b	0,00a

AN/A - Before of storage .

* Means with the same letter are not significantly different according with Tukey test (P < 0.05)

Table 3. Media number of conidia x 10^7 produced in granules (size 23,75mm^2) of *B. bassiana and M. anisopliae* mycelium *f*ormulations. 35±1^{o}C and 12 h light

Formulations	AN/A	Days of storage			
		15	30	60	90
B. bassiana					
Dry mycelium	9,43bc*	11,33ab	10,73ab	10,33a	0,00b
Sodium alginate	17,00a	15,07a	8,69bc	0,00b	0,00b
Corn starch	12,00abc	11,97a	16,33a	10,13a	7,43a
M. anisopliae					
Dry mycelium	6,90c	0,00c	0,00d	0,00b	0,00b
Sodium alginate	15,00ab	0,00c	0,00d	0,00b	0,00b
Corn starch	7,30c	5,53bc	2,53cd	0,37b	0,00b

AN/A - Before storage.

- Means with the same letter are not significantly different according with Tukey test (P < 0.05)

With regards to the last one, the results differ from those of Rombach *et al*. (1988), which showed a loss of viability of dry *B. bassiana* mycelium after a week at a temperature of 35°C. At the 60th day of storage only the treatments with corn starch and with dry *B. bassiana* mycelium became viable. On the 90th day, only the formulation with *B. bassiana* with corn starch was viable, becoming non-viable on the 120th day.

These results indicated that the preparations with the *B. bassiana* mycelium were the ones which produced the most conidia in all the situations. These are in agreement with the results of Pereira (1987) and Pereira & Robberts (1991).

Spore productions by formulations in the field.

In this experiment, information regarding the capacity of spore production of the particles of the formulated fungi were registered, as well as the loss suffered by them due to predation and other factors. The results of spore production (+), no spore production (-), germination (o) and losses (*) of the fungi particles are presented in the Table 4. As can be seen, the mycelial particles placed on the soil and plant before storage had a normal spore production in their majority, with the exception of some samples placed on the sand. After 15 days of storage at 25±1°C, there was only spore production for the treatments with *B. bassiana* and formulation of *M. anisopliae* with corn starch placed on the soil in the lab and field. On the 30th day, all the treatments with *B. bassiana* placed on the soil both in the lab and in the field had good sporulation. However, only one sample of dry mycelium sporulated on the plant. With regards to *M. anisopliae*, there was only spore production in the formulation with corn starch on the soil in the lab. After 60 days of storage, no formulation with *M. anisopliae* spored and, on the 90th day, there was only spore production for the formulations with *B. bassiana* with sodium alginate on the soil in the lab, in the field and on the plant and corn starch on the soil and the laboratory and in the plant. The excess of humidity in the soil and low temperature in the field, which took place during the sampling on the 60th day of storage, might have negatively changed the spore production of the M. anisopliae mycelium, as it was verified by Krueger *et al (*1991).

With regards to the loss of mycelial particles, it was observed that from the 168 samples taken to the field, only 23 did not recovered, corresponding to 13.69% (Table 4). This loss therefore, should not affect the performance of these preparations in the field and the formulations with mycelia represented yet another potential way for the use of these fungi.

These results showed that the *B. bassiana* is more adequate for the formulations with dry mycelium for it and resisted the storage conditions in the field better, as reported by Pereira (1987). The results also indicated that the pre-gelatinized corn starch was more appropriate than the sodium alginate for formulations with mycelia of the *B. bassiana* and *M. anisopliae* fungi.

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RESUMO

Este estudo visou a elaboração de formulações experimentais com micélios de *Beauveria bassiana* e *Metarhizium anisopliae*. Os experimentos foram desenmvolvidos no laboratório de Patologia de Insetos do Departamento de Entomologia da Escola Superior de Agricultura Luiz de Queiroz, em Piracicaba, São Paulo, Brasil. As formulações foram preparadas com alginato de sódio e amido de milho pregelatinizado e submetidas às temperaturas de 25,30 e 35^{o}C 1^{o}C. O amido de milho pregelatinizado foi mais adequado que o alginato de sódio para elaboração de formulações com micélios de *B. bassiana* e *M. anisopliae.*

Table 4. Sporulation and loss of particles of mycelium of *B. bassiana* and *M. anisopliae* in sugar cane plants and soil. 25±1°C and 12 h of light (Piracicaba, SP)

Formulations	Condition	AN/A	Days of storage			
			15	30	60	90
B. bassiana						
Dry mycelium	Lab soil	+++	+++	+++	+++	---
	Plant	+++	+++	--+	--+	--*
	Field soil	+++	++*	+++	+**	-**
Sodium alginate	Lab soil	+++	+++	+++	+++	++-
	Plant	+++	+++	--o	*-*	+--
	Field soil	+++	++*	+++	--*	++-
Corn starch	Lab soil	+++	+++	+++	+++	+++
	Plant	+--	--+	---	--*	--*
	Field soil	+++	++*	++*	--o	--*
M. anisopliae						
Dry mycelium	Lab soil .	+++	---	---	---	---
	Plant	**-	--*	---	---	---
	Field soil	+++	--*	---	--*	---
Sodium alginate	Lab soil .	+++	---	---	---	---
	Plant	++-	--*	---	--*	---
	Field soil	+++	--*	--o	-o*	---
Corn starch	Lab soil	+++	+++	+++	---	---
	Plant	++-	---	---	---	---
	Field soil	+++	++-	---	--*	---

(+) Sporulation; (-) No sporulation ; (o) Germination ; (*) Loss.
The signal refere to three replications

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