Reception: **April 1, 2023** | Acceptance: **July 7, 2023**| Publication: **October 1, 2023**

# Antifungal Effect Of Encapsulated *Metarhizium anisopliae* Spores on the Development of Bean Plants (Phaseolus vulgaris L) Infected with *Fusarium moniliforme*

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To quote this article:

Cruz Rodríguez, R. I., Porraz Ruiz, M. L., Hernández Garduza, S., & Gómez Salinas, J. A. Efecto antifúngico de esporas encapsuladas de Metarhizium anisopliae sobre el desarrollo de plantas de frijol (Phaseolus vulgaris L) infectadas con Fusarium moniliforme. *Espacio I+D, Innovación más Desarrollo, 12*(34). https://doi.org/10.31644/IMASD.34.2023.a01

*— Abstract—*

The annual bean (*Phaseolus vulgaris* L.) crop production in Mexico is reduced mainly by diseases and pests. An alternative for biological control is the use of *Metarhizium anisopliae* spores due to their ability to colonize the rhizosphere, but their effectiveness decreases due to sensitivity to environmental factors. Therefore, the objective of this study was to analyze the biocontrol potential of an alginate formulation with spores of *M. anisopliae* in the bean crop with Fusarium infected. A completely randomized experimental design was proposed using healthy plants as control; plants were also inoculated with a suspension of Fusarium oxysporum in the substrate, and additionally to a part of these plants, 1 g of capsules of alginate with M. anisopliae spores per plant was deposited at the stem base, the morphometric parameters were measured every eight days for eight weeks, as well as the appearance of Fusarium wilt symptoms. After analyzing the data obtained, it was shown that there were significant differences 64 days after inoculation (ddi), with greater leaf and root dry weight, as well as root length in plants where there were interactions between Fusarium and encapsulated spores of Metarhizium, which suggests that the presence of the microorganisms positively affected the treatment of the plants, surpassing the benefits of the application of Metarhizium reported as a growth promoter. In addition, the incidence of the root disease will occur during the first 32 days of cultivation. It was concluded that the application of encapsulated spores of M. anisopliae is a viable option as a biocontrol to reduce the effect of Fusarium wilt in the bean crop.

**Keywords:**

*Encapsulated; Metarhizium anisopliae; alginate; disease incidence.*

Due to their high protein content, beans are considered one of the main foods of gastronomy in Mexico for both the rural and urban populations, so it is vital to maintain sufficient production to meet demand since various factors influence the quality of the grain, such as agronomic management and cultivation and storage conditions, as well as being threatened by pests and diseases during its development in the field. The Fusarium species are the most aggressive, which limits the productivity of the common bean, mainly *Fusarium solani* (Toghueo *et al*., 2016). The main symptom of fusariosis is root rot, which begins with the reduction of the root system in length and weight, thus decreasing the ability of the plant to absorb nutrients and water, leading to its death. Another genus that affects this crop is Rhizoctonia, which can cause serious damage in susceptible varieties, up to 94% of the incidence of the disease and 39% in resistant varieties (Ketta & Hewedy, 2021). The conventional management to control these pathogens is the application of chemical fungicides, and the abuse of these compounds has affected other components of the ecosystem, such as the inhibition of pollinators and the reduction of beneficial microbial communities in the soil, even causing the appearance of resistant pathogens. In addition, the accumulation of these inorganic compounds affects the health of the user and the environment. In recent decades, alternatives have been sought to reduce these effects, the most promising being the use of broad-spectrum antagonist biocontrol agents (bacteria and fungi) (Uzman *et al*., 2019; Sánchez-Rodriguez *et al*., 2016).

It has been shown that non-pathogenic microorganisms associated with the plant generally protect it through rapid colonization and, therefore, use the available substrates, being limited for pathogenic organisms hindering the growth of the latter (Mayerhofer *et al*., 2019). Entomopathogenic fungal species such as *Beauveria bassiana* and some belonging to the genus Metarhizium have been used to control insects in food-important crops, they can colonize their roots (Ahmad *et al*., 2020; Razinger *et al*., 2020; Krell *et al*., 2017), promote growth and even increase their resistance to viral and fungal pathogens (Shaalan *et al*., 2021; Shaalan & Ibrahim, 2018) due to their ability to produce bioactive molecules by their secondary metabolism such as hormones and antibiotics, (Shah *et al*., 2022). The entomopathogen *M. anisopliae* naturally attacks insects of various orders (Yousef *et al*., 2018) by combining hydrolytic enzymes such as glucanases, lipases, amylases, chitinases and toxic metabolites such as destruction and cytochalasins. Unfortunately, its prevalence in the environment is limited because it is sensitive to environmental factors such as temperature, humidity, and radiation (Putnoky-Csicsó *et al*., 2020), reducing its effectiveness in crops; therefore, to prolong the benefits provided by the interaction of *M. anisopliae* with its host plant, it requires coverage that allows it to increase

its permanence and environmental resistance. In this regard, research has been carried out that proposes the inclusion of spores of the microorganism in a non-toxic polymeric matrix (Manzanarez-Jiménez *et al*., 2023) and is biocompatible. Sodium alginate is a polymer that has been used for the encapsulation of cells, which has allowed the release of cells under specific conditions and has achieved 80 to 95% survival (Sarma *et al*., 2023; De Oliveira Lopes *et al*., 2020), being a good alternative for the protection of fungal biocontrol agents, including entomopathogens (Lei *et al*., 2022). Therefore, the objective of this work was to analyze the biocontrol potential of an alginate formulation with encapsulated *M anisopliae* spores in the cultivation of beans (*Phaseolus vulgaris* L.). infected with Fusarium

## 2. MATERIALS AND METHODS

### *2.1 Microorganisms*

*Metarhizium anisopliae* was obtained from the National Institute of Agricultural and Livestock Forestry Research (INIFAP), and *Fusarium moniliforme* was molecularly identified (no.GU982311.1).

### *2.2 Encapsulation of Metarhizium anisopliae spores in sodium alginate*

The alginate capsules were prepared by ionic gelation, as reported by Meirelles *et al*., (2023) with some modifications. The 4% p/v sodium alginate solution (Sigma-Aldrich-Merck brand) was prepared and sterilized at 15 Lb for 15 minutes. Subsequently, in a laminar flow hood, that solution was mixed with a spore suspension ($1x10^7$ spores/mL.) of *M. anisopliae*, which was prepared from a 7-day Czapek broth culture at 4% v/v. It was kept under agitation until the mixture was uniform and deposited into a 20 mL needleless syringe, from which it was dropped dropwise onto a solution of 10% calcium chloride ($CaCl_2$) (Meyer Brand) previously sterile and at room temperature that was kept under continuous stirring, the mixture (Na-spore alginate) upon contact with the CaCl2 solution instantly forms the beads, encapsulating the spores. The beads or capsules were kept in the $CaCl_2$ solution for 30 min, after which time they were washed with sterile water and placed in a dry and sterile container, finally dried in an oven at 45 °C for 48 h.

### *2.3 Plant material*

The bean seeds were obtained from the greenhouse of the Tecnológico Nacional de México, Tuxtla campus from preservation crops.

### *2.4 Establishment of the experiment at the greenhouse level*

From a previous culture in Petri dishes with potato dextrose agar (PDA) for seven days at 28 ± 2°C, the pathogen inoculum suspension was prepared at a suspension concentration of 1x10$^6$ spores/mL *F. moniliform*, for which 10 mL of sterile distilled water and glass beads that were slowly stirred were added to each plate to detach the spores from the mycelium. Each bean seed was sown in 100 g of Peat moss substrate in black nursery bags at a 4 cm depth; 12 days after germination, the plants were transplanted into pots containing 500 g of the same substrate, and four treatments were evaluated: 1) bean - neutral control -(Fr), 2) bean + *F. moniliform* (Fr + Fm), 3) beans + capsules of *M. anisopliae* (Fr + CMa), 4) beans + capsules of *M. anisopliae* + *F. moniliform* (Fr + CMa + Fm). At the time of transplanting for treatments 3 and 4, 1 g of M. anisopliae spore capsules were deposited at a distance of no more than 5 cm from the base of the stem. 7 days after the bean plants had contact with the M. anisopliae capsules, plants from treatments 2 and 4 were inoculated with 20 mL of a suspension of *F. moniliform*. The development of symptoms of fusariosis was analyzed using six plants per treatment; observations were made at 15-day intervals for two months. The variables evaluated were fresh weight and leaf and root dry weight (g), root length (cm), plant height (cm), chlorophyll (SPAD), stem diameter (mm), and root incidence (%).

For each experimental unit, the variables stem diameter, height, and root length were measured with a vernier and/or flexometer, to obtain the fresh and dry weights, the plants were removed from the substrate, washed, and dried with absorbent paper and separated into two sections: root and leaf, then weighed on an analytical balance and deposited in paper bags and dried in an oven at 45ºC until constant dry weight values were obtained.

The percent disease incidence (IR) in roots was determined by the following formula: IR (%) =n/N x 100. Where n is the number of plants showing disease symptoms with at least one diseased root, and N is the total number of samples used (Cruz-Rodriguez *et al.*, 2020).

### *2.5. Experimental design and statistical analysis*

A randomized complete block design was used, four blocks with six repetitions monitored four times; with a total of 96 experimental units. For statistical analyses, one-way ANOVA at a 5% significance level was used using the Statgraphics program.

## 3. RESULTS AND DISCUSSION

### *Effect of M. anisopliae encapsulates on the growth parameters of bean plants*

The application of capsules with *M. anisopliae* spores had a positive effect on leaf and root system development in bean plants at 64 days after inoculation (ddi) (Table 1). The parameters focused on root growth such as fresh and dry weight, in addition to length, are those that mainly show variations between treatments 1 and 2, the latter being the one in which the lowest values of the entire experiment are observed because it is the tissue where the Fusarium infection process begins. In the case of treatment 4, which was also inoculated with *F. moniliform*, this presents not only significant differences in root growth with the rest of the treatments but also in leaf development, such is the case of the number of leaves with this treatment (66 leaves), a 55% increase was observed concerning the plants of treatment 1, that developed under normal conditions with an average value of 42.5 leaves.

Some of the processes that result from the interaction of the cells of the entomopathogenic fungus with the plant cell tissue, initiate through the production of hydrolytic enzymes by the fungus, which allow the degradation of the components of the plant cell wall and, this way, the hyphae can penetrate the surface of the cells thus colonizing the roots, facilitating the uptake of nutrients and water (Liao *et al*., 2013). Another of the reported mechanisms of the genus Metarhizium is its ability to activate defense signaling pathways in the plant cell that results in the production of molecules such as phytohormones that accelerate growth. This was observed in *Arabidopsis thaliana* plants, due to the presence of *Metarhizium robertsii*, through an increase in the production of Indole Acetic Acid (IAA), which promoted the development of root hair (Liao *et al*., 2017). Finally, the cells of the entomopathogenic fungus can act as an elicitor, to accelerate the production of phytochemicals that protect the cell from F. moniliform *attack*, and reduce symptoms in plants due to the presence of the pathogen.

The presence of *F. moniliform* significantly affected chlorophyll production in treatments 2 and 4 with values of 29.84 + 4.72 and 31.7 + 0.12 respectively, however, it did not present significant differences with treatment 1, while the interaction of the entomopathogen with the plant in treatment 3, showed the highest relative index of chlorophyll with 40.41 + 4.24, this index is expressed in SPAD units and has been positively correlated with the state or nitrogen absorption capacity of the plants, Casierra *et al*., (2012) point out that the content of photosynthetic pigments can increase or decrease due to stress factors (light, temperature, humidity, nutrients, etc.), to the physiological age or photosynthetic capacity of the plant, in this case, the variation is due to biotic stress.

*Analysis of the incidence of disease caused by Fusarium moniliform*

Fusarium species are known as causative agents of root and stem wilt and rot, one of the first symptoms is browning of the root tissue due to the infection mechanism of these pathogens and at an advanced level chlorosis, vascular discoloration and decay are observed, for this reason the monitoring of the incidence in the root (IR) was carried out during the 64 days that the experiment lasted, as seen in Figure 1, the percentage of IR was presented in both treatments where the pathogen was inoculated, at the 8 days after inoculation (16 ddi), the percentage of incidence increased as the crop cycle progressed, with the incidence being higher in all samples of treatment 3, reaching an IR of 25.8 + 0.56 % and after 48 days a decrease in the presence of Fusarium necrosis was observed up to 14.6 + 4.6 %. In the case of treatment 4 plants, which were pre-treated with *M anisopliae* capsules showed slight resistance in the first days of infection, 3.2 + 0.4 % at 16 days and reaching a maximum incidence of 8.6 % at 64 days of the entomopathogen, which is not statistically different from the IR of treatment 3 at 16 ddi. This shows that the presence of *M anisopliae* markedly decreased the normal development of the disease; making the entomopathogenic plant-fungus symbiosis effective against Fusarium. It is suggested that growth inhibition mechanisms against *F. moniliform* include the combination of the activity of hydrolytic enzymes produced by the entomopathogen, which act on the cell wall of Fusarium together with the induction of phytoalexins by bean plants (García-Enciso *et al*., 2018).

## CONCLUSION

The formulation of alginate to encapsulate the spores of *Metarhizium anisopliae* spores allowed their release into the soil, being indirectly evident by the growth-promoting effect on bean plants, it was also shown that the interaction of the spores of the entomopathogen with the plant tissue reduces the incidence of *F moniliform* in the root. Therefore, the addition as a pretreatment of capsules with spores of *Metarhizium anisopliae* is an option to control fusariosis in bean cultivation, and thereby reduce in the future the application of chemical substances in the field for the control of diseases in crops caused by fungi.

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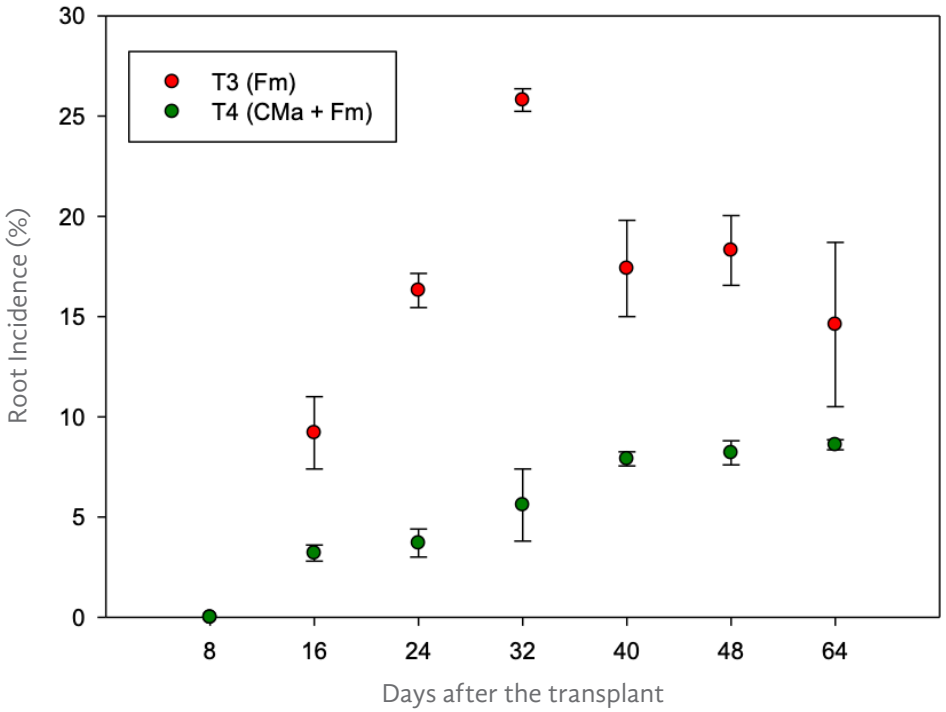


*Figure 1.* Percentage incidence of fusarium root blight in Phaseolus vulgaris L. Fm: F. moniliforme. CMa: M. anisopliae spore capsules

**Table 1**

*Morphometric variables of bean (Phaseolus vulgaris L). plants infected with Fusarium 64 days after inoculation*

| | | Variables | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **No.** | **Treatment** | Fresh leaf weight (g) | Dry leaf weight (g) | Fresh root weight (g) | Dry root weight (g) | Root length (cm) | Number of leaves | Plant height (cm) | Relative Chlorophyll Index (SPAD units) |
| 1 | Fr | 12.97 ± 4.63 c | 2.48 ± 0.50 c | 2.47 ± 0.21 c | 0.54 ± 0.02 c | 27.1 ± 0.22 c | 42.5 + 0.71 b | 60.4 + 0.71 ab | 34.94 + 1.18 b |
| 2 | Fr + Fm | 16.35 ± 4.75 c | 3.11 ± 0.63 c | 2.08 ± 0.12 d | 0.48 ± 0.03 d | 26.4 ± 0.0 d | 45.0 + 8.49 b | 45.0 + 8.49 c | 29.84 + 4.72 bc |
| 3 | Fr + CMa | 25.81 ± 0.0 b | 4.68 ± 0.93 b | 3.38 ± 0.42 b | 0.66 ± 0.05 b | 29.04 ± 1.07 b | 48.5 + 9.31 b | 75.75 + 17.04 b | 40.41 + 4.24 a |
| 4 | Fr + CMa + Fm | 48.05 ± 0.0 a | 9.46 ± 0.0 a | 6.49 ± 0.0 a | 1.13 ± 0.0 a | 34.29 ± 0.77 a | 66.0 + 1.21 a | 110..0 + 2.5 a | 31.7 + 0.12 c |

Mean values of three replicates followed by at least one letter, which are not significantly different at P < 0.05 (Tukey's test). **Fr: bean; CMa: M. anisopliae spore capsules; Fm: F. moniliforme.**